Submission to the French
Commission du Génie Biomoléculaire

Application to Place on the Market Genetically Modified Higher Plants:

Insect-Protected Maize
(MON 810)

by

Monsanto Company represented by Monsanto Europe S.A.

List of Abbreviations

2,4-D	(2,4-dichlorophenoxy)acetic acid
APHIS	Animal Plant Health Inspection Service
bp, Kb	Base pairs, kilobase pairs
B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
CaMV	Cauliflower mosaic virus
CaMV E35S	35S promoter with enhancer sequence from CaMV
CFR	U S. Code of federal regulations
CP4 EPSPS	EPSPS from *Agrobacterium* sp. strain CP4
cryIA(b)	Class I (Lepidoptera-specific) crystal protein gene
CTP	Chloroplast transit peptide
E. coli	*Escherichia coli*
ECB	European corn borer
ELISA	Enzyme-linked immunosorbent assay
EPA	U.S. Environmental Protection Agency
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
FDA	U.S. Food and Drug Administration
GLP	Good Laboratory Practices
GMO	Genetically Modified Organism
gox	Gene for glyphosate oxidoreductase
GOX	Glyphosate oxidoreductase
hsp70	Intron sequence from maize heat-shock protein 70
IPM	Integrated Pest Management
kD	Kilodaltons
N.A.	Not analysed
N.D.	Not detected
NOS 3'	3' transcriptional termination sequence from nopaline synthase
NPTII	Neomycin phosphotransferase II
nptII	Gene for neomycin phosphotransferase II
ori-pUC	Bacterial origin of replication from the pUC plasmid
PCR	Polymerase chain reaction
ppm	parts per million
SSUIA	small subunit gene of ribulose-1,5-bisphosphate carboxylase
USDA	United States Department of Agriculture
w/w	weight/weight

Table of Contents

PART A: GENERAL INFORMATION

PART B: NATURE OF THE GMOS CONTAINED IN THE PRODUCT

INFORMATION RELATING TO THE RECIPIENT OR PARENTAL ORGANISM(S) FROM WHICH THE GMO IS DERIVED

INFORMATION RELATING TO THE GENETIC MODIFICATION

INFORMATION ON THE ORGANISM(S) FROM WHICH THE INSERT IS DERIVED (DONOR)

INFORMATION RELATING TO THE GMO(S) CONTAINED IN THE PRODUCT

INTERACTIONS OF THE GMO WITH THE ENVIRONMENT

PART C: PREDICTED BEHAVIOUR OF THE PRODUCT

List of Supporting Information

Figures

Tables

A. GENERAL INFORMATION

1. Details of notification

(a) Member State of notification: France

(b) Notification number:

(c) Name of the product (commercial and other names):

The products which are the subject of this application are seeds of Insect-Protected maize line MON 810 and seeds of any progeny (inbreds or hybrids) derived from this line by conventional breeding methods. The application addresses the production of Insect-Protected maize in the European Union, the import and processing of grain and maize products produced from Insect-Protected maize and their eventual use in food, feed and industrial products.

(d) Date of acknowledgement of notification:

2. Notifier/manufacturer/importer

(a) Name of the notifier: Monsanto Company represented by Monsanto Europe S.A.

(b) Address of the notifier:

Monsanto Europe
270-272 Avenue de Tervuren
B-1150 Brussels
BELGIUM

Monsanto Company
800 N. Lindbergh Boulevard
St. Louis, Missouri 63167
U.S.A.

(c) The notifier is: domestic manufacturer and developer of the gene technology in maize line MON 810.

(d) In case of import

(i) Name of the importer:
(ii) Address of the importer:

3. *Characterization of the GMOs contained in the product*

Indicate the name and nature of each type of GMO contained in the product

Insect-Protected maize line MON 810 expresses the CryIA(b) protein derived from *Bacillus thuringiensis* subsp. *kurstaki*. The products which are the subject of this application are seeds of Insect-Protected maize line MON 810 and seeds of any progeny (inbreds or hybrids) derived from this line by conventional breeding methods. At the time of market introduction, a commercial name (trademark) for Europe will be given to the Insect-Protected maize, in addition to the names of subsequent commercial hybrids.

The plants of maize line MON 810 and varieties (inbreds or hybrids) derived from this line by conventional breeding methods, produce a CryIA(b) insect control protein (Höfte and Whiteley, 1989*) derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). The CryIA(b) protein encoded by the vector used to produce Insect-Protected maize varieties is identical to the protein found in nature (Fischhoff *et al.*, 1987*; Lee *et al.*, 1995b) and in commercially registered microbial formulations (Lee *et al.*, 1995a).

Insect-Protected maize plants, developed by Monsanto in collaboration with maize breeders, provide effective control of European corn borer (*Ostrinia nubilalis*), an economically damaging insect pest in European and U.S. maize (Gay, 1993; USDA, 1992). Depending on the geography, European corn borer (*Ostrinia nubilalis*) may have from one to three generations during the growing season of maize. European corn borer (ECB) damage to maize plants includes: (1) leaf feeding (from the first generation), (2) stalk tunnelling (from the first and second generations), (3) leaf sheath and collar feeding (from the second and third generations), and (4) ear damage (from the second and third generations). Chemical insecticides offer limited utility as applications must be made prior to the time the insect bores into the stalk and repeat applications are often necessary. The use of Insect-Protected maize will enable farmers to effectively control ECB, providing yield protection and a reduction in the use of chemical insecticides for this insect pest. Insect-Protected maize will provide benefits to growers, the general public, and the environment, including: (1) a more reliable, economical, and less labour intensive means to control ECB, (2) insect control without harming non-target species, (3) a means for growers to significantly reduce the amount of chemical insecticides now applied to the crop thereby achieving ECB control in a more environmentally compatible manner than is currently available, (4) a reduction in the manufacturing, shipment, and storage of chemical insecticides used in maize, (5) a reduction in the exposure to workers to the pesticide and pesticide spray solution, (6) a reduction in the number of empty pesticide containers and amount of spray solution that must be disposed of according to applicable environmental regulations, (7) a fit with integrated pest management (IPM) and sustainable agricultural systems, and (8) both large and small growers will benefit from the planting of Insect-Protected maize as no additional labour, planning, or machinery is required.

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* These references have been provided in a separate volume to the French Commission du Génie Biomoléculaire

4. General description of the product

(a) Type of product

The products which are the subject of this application are seeds of Insect-Protected maize line MON 810 and seeds of any progeny (inbreds or hybrids) derived from this line by conventional breeding methods. These seeds will be marketed as new maize varieties and grain and maize products derived from these varieties will be introduced into commerce like any other maize variety. Insect-Protected maize line MON 810 has been genetically modified to express the CryIA(b) insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). Expression of the protein in the plant provides control of European corn borer and certain other lepidopteran insect pests such as pink borer (*Sesamia cretica*).

(b) Composition of the product

Insect-Protected maize line MON 810 and varieties (inbreds or hybrids) derived from this line by conventional breeding methods are substantially equivalent to other maize varieties with the exception that they produce one additional protein in low concentrations, the CryIA(b) insect-control protein derived from *Bacillus thuringiensis* subsp. *kurstaki* (Höfte and Whiteley, 1989*).

c) Specificity of the product

For the users of Insect-Protected maize seed there are no specific differences compared to other maize varieties except for the control of ECB. The only difference is that the insect control practices used during cultivation are modified. The seed, grain and maize product uses are identical to those for seed, grain and maize products from other maize varieties.

(d) Types of users

This application addresses the production of Insect-Protected maize in the European Union and the import, storage and processing of grain and maize products produced from Insect-Protected maize and their use in feed, food and industrial products by downstream users including traders and processors.

Insect-Protected maize will be used in a manner consistent with current uses of maize grain and grain products. The primary use of maize is for animal feed. 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 greatest volume is processed by wet milling to produce starch and sweetener products for foods. Nonfood products such as industrial starches, corn gluten feed, and corn gluten meal are also manufactured (May, 1987; Watson, 1988). The primary products derived from the dry milling process are maize grits, maizemeal, and maize flours. The largest food/feed product volume of the

dry-milling industry is animal feed followed by brewing and food uses (Rooney and Serna-Salvidar, 1987). Virtually all processing of maize and dry milled fractions into finished food products involves exposure to high temperatures.

(e) Exact conditions of use and handling

The seed, grain and grain products of Insect-Protected maize will be used and handled in the same manner as current commercial maize varieties.

The cultivation of Insect-Protected maize may be combined with the use of non insect-protected maize, as part of a strategy to prevent the occurrence of European Corn Borer resistance to the CryIA(b) protein (see section C, paragraph 1-e).

(f) Geographical areas for which the product is intended

Insect-Protected maize varieties will be grown in major maize producing areas of the world including Africa, North America, South America, Asia and Europe, particularly in France, Italy and Germany. The maize area distribution in the E.U. is presented in Table A.1. The European corn borer (*Ostrinia nubilalis*) and the pink borer (*Sesamia cretica*) infested areas are primarily the Centre-West and the South of France, Northern Italy, Spain and more marginally southern Germany. This Western European infested area is estimated to 2 to 2.3 millions ha.

Table A.1. Maize Area Distribution in the European Union (in kha)

Countries	Grain maize area (93)	Silage maize area (93)	Total maize area (estimate)
France	1860	1460	3320
Germany	330	1210	1540
Italy	930	320	1250
Spain	275	114	390
Austria	170	101	271
Netherlands	12	223	235
Greece	210	-	210
Belgium-Lux	26	164	190
Portugal	170	6	176
U.K	-	78	78
Denmark	-	20	20
Ireland, Finland, Sweden	-	-	-
Total E.U. 15	3983	3696	7679

(g) Type of environment for which the product is suited

Maize, because of its many divergent types, may be grown over a wide range of climatic conditions (Shaw, 1988). Insect-Protected maize can be produced in any environment suited to the production of maize dependant upon specific varietal germplasm.

(h) Annual estimated production in and/or imports into the Community

Production: Maize production in Europe represents a significant portion of world maize production. Although the United States produces the largest percentage of the world maize production, two regions in Europe are among the top five Corn Belts in the world. These are the Danube Basin from Southwest Germany to the Black Sea along with Southern France through the Po Valley of Northern Italy. Figures from 1994 show total production of maize within the fifteen countries comprising the EU to be over 28 millions tons (Table A.2). The largest producer was France with over 12 millions tons, followed by Italy with 7.4 million tons. In total, 30% of the total acreage in France for corn is irrigated and yields average eight tons per hectare. Very good growing conditions for maize in Italy result, on average, in yields of eight tons per hectare.

Imports: In 1994, 2.5 millions tons were imported from outside the E.U. (Table A.2).

Table A.2. Maize Production and Imports within the E.U.

Supply balance sheet for maize (October 1994 - September 1995). Figures are in thousand tons. Imports from countries outside Europe as well as imports between European countries.

	Germany	Belgium Luxemb.	Denmark	Spain	France	Greece	Ireland	Italy
Stock intervention	209	0	0	0	694	0	0	53
Stock free harvest 93	58	109	7	131	-333	-155	25	-504
Production 94	2357	106	0	2222	12640	1800	0	7400
Imports intra EU	781	1123	72	712	83	64	39	579
Imports third country	197	20	4	1382	68	18	0	38
Supply	3602	1358	83	4447	13152	1727	64	7566
Exports intra EU	236	403	0	32	5965	103	1	13
Exports third country	2	0	0	0	119	0	0	0
Animal consumption	1916	263	21	3737	4676	1489	40	7020
Seed and losses	150	1	3	28	200	25	0	43
Demand	3504	1297	76	4597	12970	1777	56	7866
Stock Oct. 95	150	61	6	100	798	220	8	600
Surplus/shortage	0	0	0	0	299	148	0	0

	Netherlands	Portugal	United Kingdom	Austria	Finland	Sweden	EU12	EU15
Stock intervention	0	0	0	0	0	0	956	956
Stock free harvest 93	74	136	42	6	0	0	-410	-404
Production 94	114	555	0	1421	0	0	27194	28615
Imports intra EU	1731	435	1293	0	0	0	6912	6912
Imports third country	53	500	217	8	0	0	2497	2505
Supply	1972	1626	1552	1435	0	0	37149	38584
Imports intra EU	147	0	12	0	0	0	6912	6912
Exports third country	0	0	0	23	0	0	121	144
Animal consumption	830	1417	180	1278	0	0	21589	22867
Seed and losses	0	5	0	3	0	0	455	458
Demand	1877	1602	1537	1370	0	0	37159	38529
Stock Oct. 95	96	124	15	65	0	0	2178	2243
Surplus/shortage	0	0	0	0	0	0	446	446

Source Stratégie Grains. Analyse et prévision du prix des céréales Menuel, no 27, 15 Mars 1995 (France)

5. *Has the combination of GMOs contained in the product been notified under part B of Directive 90/220/EEC?*

Yes X No

(i) If yes, give country and notification number:

Table A.3. Insect-Protected Notifications in the European Union

Year	Country	Notification Number	Authorized by
1994	France	94.02.11	Ministry of Agriculture
		94.02.16	Ministry of Agriculture
		94.03.02	Ministry of Agriculture
1995	France	95.03.06	Ministry of Agriculture
		95.03.08	Ministry of Agriculture
		95.03.09	Ministry of Agriculture
		95.03.10	Ministry of Agriculture
		95.03.11	Ministry of Agriculture
		95.03.12	Ministry of Agriculture
	Italy	B/IT/95-38	Ministry of Health
		B/IT/95-23	Ministry of Health

6. *Is the product being simultaneously notified to another Member State?*

Yes No X

7. *Has another product with the same combinations of GMOs been placed on the EC market by another notifier?*

Yes No X Not known

8. *Information on releases of the same GMOs or of the same combination of GMOs previously or currently notified and/or carried out by the notifier either inside or outside the Community*

Insect-protected maize line MON 810 has been tested:
- in France and Italy since 1994 in over 18 test sites to date.
- in the U.S. since 1993 in over 60 test sites to date documented under USDA APHIS final reports. USDA APHIS regulations under 7 CFR 340.2 regulate the import, release and interstate movement of genetically modified organisms.
- in Chile, Argentina, Canada, and South Africa since 1993. Approvals to import seed and conduct field trials were provided by appropriate governmental

agencies within these countries.

All trials were conducted under supervision of Monsanto or seed company cooperators Trials were monitored on a regular basis during crop development and after crop harvest. Growth and development of Insect-Protected maize was judged to be comparable to that of other maize varieties.

Table A.4. Ex-E.U. Insect-Protected Field Trials

Year	Country	Approval Number	Approved by
1993	U.S.	93-012-04, 93-245-02N, 93-258-04N, 93-279-04N, 95-308-02N	USDA/APHIS
	Chile	2516	(S.A.G) Ministry of Agriculture
1994	U S	524-EUP-82 (245 ha)	EPA
	Chile	S.A.G. 2722	Ministry of Agriculture
	Chile	S.A.G. 2886	Ministry of Agriculture
	Argentina	716/94	CONABIA/IASCAV
	South Africa	14/2/2/1(3/94/143)	Director. Plant Quality Control
1995	U.S.	524-EUP-82 extended (569 ha)	EPA
	Canada	94-PHI1-CORO1-ON-32-01	Agriculure and Agri-food
	Chile	S.A.G. 2730	Ministry of Agriculture
	Chile	S.A.G. 2728	Ministry of Agriculture
	South Africa	16/2/21(3/95/130)	Director. Plant Quality Control

9. Specify instructions and/or recommendations for storage and handling

Insect-Protected maize has been demonstrated to be substantially equivalent to other maize, apart from its protection from certain lepidopteran insects, so no specific instructions or recommendations for storage or handling of the seeds, grain or derived products from Insect-Protected maize are envisaged. Grain from Insect-Protected maize will be mixed with other maize varieties following harvesting. as with any other new maize variety.

10. Proposed packaging

Insect-Protected maize varieties have been shown to be substantially equivalent to other maize varieties in growth characteristics, yield, survival, composition and other parameters, so the existing handling and packaging systems for maize varieties will apply without need for changes. Insect-Protected maize in the form of grain and processed products will be used in the same manner as grain and grain products from other maize varieties.

11. Proposed labelling

Insect-Protected maize varieties will be identified to allow growers to know they are purchasing Insect-Protected maize seed. At the time of market introduction, a commercial name (trademark) for Europe will be given to the Insect-Protected maize, in addition to the variety names of the commercial hybrids.

After harvest, Insect-Protected maize in the form of grain as a raw material and processed products will be marketed in the European Union without any specific labelling since 1) these products have been shown to be substantially equivalent to other maize grain products and will be marketed in mixtures with materials from other maize varieties and 2) the new agronomic trait does not distinguish these varieties for processors or other users of these maize products.

12. Measures to take in case of unintended release or misuse

Insect-Protected maize has been shown to be substantially equivalent to other maize. Maize is not an invasive plant because it is a weak competitor outside the cultivated fields and seed dispersal of individual kernels is naturally limited because of the structure of the ears of maize. Maize volunteers are easily controlled by current agronomic practices including cultivation and the use of selective herbicides currently used in the rotation. Therefore, no specific measures are recommended in case of unintended release of Insect-Protected maize.

13. Measures for waste disposal and treatment

The measures for waste disposal and treatment for Insect-Protected maize products are the same as those for other maize products.

B. NATURE OF THE GMOS CONTAINED IN THE PRODUCT

INFORMATION RELATING TO THE RECIPIENT OR PARENTAL ORGANISM(S) FROM WHICH THE GMO IS DERIVED

14. Scientific name and other names

a) Family name:	Gramineae
b) Genus:	*Zea*
c) Species:	*mays* (2n = 20)
d) Subspecies:	none
e) Cultivar/line:	"High Type-II" germplasm (Hi-II)
f) Common name:	Maize; Corn

The Hi-II genetic material, the initial recipient of the added gene, is a derivative of the A188 and B73 inbred lines of maize. These are publicly-available inbred lines developed in the U.S. by the University of Minnesota and Iowa State University, respectively. Designated "Hi-II", the recipient material is approximately 50:50 mix of the two lines (Armstrong *et al.*, 1991). The material was developed to have a higher regeneration potential during the tissue culture stages along with acceptable commercial performance in hybrids.

15. Phenotypic and genetic traits

Maize (*Zea mays)* reproduces sexually. Maize is a wind-pollinated, monoecious species with separate staminate (tassels) and pistillate (silk) flowers which encourages 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 (Goss, 1968*; Kiesselbach, 1949*). Wind movements across the maize field cause pollen from the tassel to fall on the silks of the same or adjoining plants. Measuring about 0.1 mm in diameter, maize pollen is the largest of any pollen normally disseminated by wind from a comparably low level of elevation. Dispersal of maize pollen is influenced by its large size and rapid settling rate (Raynor *et al.*, 1972*). Self-pollination leads to homogeneity of the genetic characteristics within a single plant while cross-pollination combines the genetic traits of many plants. This inbred-hybrid concept and resulting yield response is the basis of the modern seed maize industry.

16. Geographic distribution and natural habitat of the organisms

Maize, because of its many divergent types, is grown over a wide range of climatic conditions. The bulk of the maize is produced between latitudes 30° and 55°, with relatively little grown at latitudes higher than 47° latitude anywhere in

the world (Shaw, 1988). The greatest maize production occurs where the warmest month isotherms range between 21 and 27° C and the freeze-free season lasts 120 to 180 days. A summer rainfall of 15 cm is approximately the lower limit for maize production without irrigation with no upper limit of rainfall for growing maize, although excess rainfall will decrease yields. Table B.1. lists the major maize production areas.

Table B.1 World Maize Production 1994-95* (Grain)

Country	Production (MMT**)	(% of total)
U.S.	256.63	46.3
China	101.00	18.8
Brazil	31.00	5.6
E.U.	26.93	4.9
E. Europe	22.39	4.0
Mexico	16.00	2.9
Argentina	10.50	1.9
India	10.50	1.9
Canada	7.05	1.3
South Africa	7.00	1.3

*National Corn Growers Association. 1995. The World of Corn.
**MMT: Million metric tons

17. Genetic stability of the organism and factors affecting it

Maize has a long history as an agricultural crop and is genetically the most accessible and characterised among the higher plants (Coe *et al.*, 1988). Precise cytological and cytogenetic experimentation has been carried out on key stages of meiosis (Carlson, 1988); various tissues and developmental stages have been defined, dissected and explored systematically; and molecular studies in maize have advanced to the point that many genes have been cloned, and several parts of the maize genome have been characterised or sequenced in detail (Coe *et al.*, 1988; Walbot and Messing, 1988). Maize is continually being modified by breeders to improve its quality and agronomic performance (Hageman and Lambert, 1988).

18. Potential for genetic transfer and exchange with other organisms

a. Outcrossing with cultivated *Zea* varieties

Maize is wind pollinated, and the distance that viable pollen can travel depends on prevailing wind patterns, humidity, and temperature. All maize will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors (Ga^s, Ga, and ga allelic series on chromosome 4). Pollen

of a specific hybrid can be carried by wind to pollinate other dent maize hybrids, sweet maize, and popcorn, if the popcorn does not carry the dent-sterile gametophyte factor (Hallauer, 1995). Maize pollen, therefore, moves freely within an area, lands on silks of the same cultivar or different cultivars, germinates almost immediately after pollination, and within 24 hours completes fertilization.

Certification standards for distances between different maize genotypes have been established to assist in the production of hybrid maize having the desired levels of purity. In Europe, the specific isolation field to produce commercial hybrid seed shall be located so that the seed parent is no less than 300 m from other maize of a similar type (200 m in the U.S.). The recommended distance for inbreds production is 400 m.

b. Outcrossing with wild *Zea* species

There are few other plant species that cross with maize, and those that do are all New World species. Maize and teosinte (*Zea mays* ssp. *mexicana* Schrad.) are genetically compatible, and in areas of Mexico and Guatemala they freely hybridise when in proximity to each other (Doebley and Iltis, 1980*). Teosinte exists primarily as a weed around the margins of maize fields, and the frequency of hybrids between teosinte and maize has been studied (Wilkes, 1972). Neither annual nor the perennial races of teosinte can survive freezing temperatures and all are adapted to short days, thus the ranges of both are usually restricted to the tropics and semi-tropics (Goodman, 1995).

Maize can be crossed with great difficulty with many of the species of *Tripsacum*, but the progeny are male sterile. *Tripsacum*-maize hybrids have not been observed in the field and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972; Goodman, 1995; Goodman 1988*). *Tripsacum* evolved by polyploidy, whereas maize and teosinte have undergone introgressive hybridization at the diploid level (2n = 20). Tripsacum species are perennials and seem to be more closely related to the genus *Manisuris* than to either maize or teosinte. Sixteen species of *Tripsacum* have been described with twelve of these species native to Mexico and Guatemala. *Tripsacum floridanum* is native to the southern tip of Florida in the U.S. while *Tripsacum australe* and two other species are native to South America.

There are few other plant species that cross with maize, and those that do are all New World species not found in Europe, except for botanical garden-like settings. Outcrossing of Insect-Protected maize with cultivated maize and wild relatives will be the same as for current maize varieties.

c. Outcrossing with other species to which it cannot interbreed

There is no published evidence for the existence of any mechanism, other than sexual crossing by which genes can be transferred from a plant to other organisms

19. Information concerning reproduction and factors affecting it

Maize is an annual crop with a cultural cycle ranging from as short as 60 to 70 days to as long as 43 to 48 weeks from seedling emergence to maturity (Shaw, 1988). This divergence in maturity allows maize to be grown over a wide range of climatic conditions. As a wind-pollinated, monoecious grass species, self-pollination and fertilization and cross-pollination and fertilization are usually possible and frequencies of each are usually determined by physical proximity and other physical influences on pollen transfer.

Tasselling, silking, and pollination are the most critical stages of maize development and grain yield is greatly impacted by moisture and fertility stress. Under conditions of high temperature (Herrero and Johnson, 1980*) and desiccation (Hoekstra *et al.*, 1989*), maize pollen viability is measured in minutes; these conditions may even blast the tassel before any viable pollen is shed (Lonnquist and Jungenheimer, 1943). More moderate conditions can extend the field life of pollen to hours (Jones and Newell, 1948*).

20. Information on survival and factors affecting it

a. Ability to form structures for survival or dormancy

Maize is an annual crop and seeds are the only survival structures. Natural regeneration from vegetative tissue is not known to occur. Modern maize cannot survive as a weed. Volunteer maize is not found growing in fence rows, ditches, and road sides as a weed. Although maize from the previous crop year can over-winter in mild winter conditions and germinate the following year, they cannot persist as a weed (Hallauer, 1995). The appearance of maize in rotational fields following the maize crop from the previous year is usually rare under European conditions. Maize volunteers are killed by frost or easily controlled by current agronomic practices including cultivation and the use of selective herbicides.

b. Specific factors affecting the capacity for survival

Maize cannot survive without human assistance and is not capable of surviving as a weed due to past selection in its evolution. In contrast to weedy plants, maize has a polystichous female inflorescence (ear) on a stiff central spike (cob) enclosed in husks (modified leaves). Consequently, seed dispersal of individual kernels naturally does not occur because of the structure of the ears of maize (Rissler and Mellon, 1993*).

Maize grain survival is dependant upon temperature, moisture of seed, genotype, husk protection and stage of development (Rossman, 1949*). Freezing temperatures have an adverse effect on maize seed germination and has been identified as a major risk in seed maize production (Wych, 1988). Temperatures above 45°C have also been reported as injurious to maize seed viability (Craig, 1977).

21. Ways of dissemination and factors affecting it

Maize is an annual crop and seeds are the only survival structures. Natural regeneration from vegetative tissue is not known to occur. Seed dissemination is impacted by mechanical harvesting and transport as well as insect or wind damage which may cause some mature ears to fall to the ground and avoid harvest. Pollen dispersal is influenced by wind and weather conditions.

22. Interactions with the environment

Maize is known to interact with other organisms in the environment including insects, birds, and mammals. It is susceptible to a range of fungal diseases and insect pests, as well as competition from surrounding weeds.

23(a) Detection techniques

Maize is a cultivated species of the Gramineae family and has been well characterised taxonomically.

23(b) Identification techniques

Maize is a cultivated species of the Gramineae family and has been well characterised taxonomically.

24. Classification under existing Community rules concerning the protection of human health and/or the environment

Not classified.

25(a) Pathogenic characteristics

Not applicable.

25(b) Other harmful characteristics of the organism living or dead, including its extracellular products

Maize is extensively cultivated and has a history of safe use. Maize is not considered to be harmful.

26. Nature and description of known extrachromosomal genetic elements

Not relevant.

27. History of previous genetic modifications

The maize from which Insect-Protected line MON 810 was developed is a combination of publicly available germplasms (see question 14 above). It has not previously been genetically modified.

INFORMATION RELATING TO THE GENETIC MODIFICATION

28. Methods used for the genetic modification

Plasmid DNA was introduced into the plant tissue by the particle acceleration method (Klein *et al.*, 1987). DNA was precipitated onto microscopic tungsten or gold particles using calcium chloride and spermidine. A drop of the coated particles was then placed onto a plastic macrocarrier, which was accelerated at a high velocity through a barrel by the explosive force of a gunpowder discharge. The macrocarrier hits a plastic stopping plate which stops the flight of the macrocarrier but allows continued flight of the DNA-coated particles. The particles penetrate the target plant cells, where the DNA is deposited and incorporated into the cell chromosome. The cells were incubated on a tissue culture medium containing 2,4-D which supports callus growth. Although the DNA solution used for transformation contained genes encoding for glyphosate tolerance (e.g., the CP4 EPSPS and *gox* genes) allowing selection of genetically modified cells on media containing glyphosate, these genes are not present in MON 810 plants. Therefore it is likely that the cell that resulted in the MON 810 line is an "escape" from glyphosate selection (with cells in the vicinity degrading the available glyphosate, for instance, allowing the cell containing the MON 810 insert to survive). In the subsequent phase, plants were regenerated from the callus tissue, in the absence of glyphosate and were assayed for the presence of the expressed CryIA(b) protein product.

29. Characteristics of the vector

(a) Nature and source of the vector

The transformation plasmids in the DNA solution used to produce Insect-Protected maize line MON 810 were PV-ZMBK07 and PV-ZMGT10. These vectors are shown in Figures B.1 and B.2 respectively.

A description of the DNA elements in PV-ZMBK07 and PV-ZMGT10 are given in Tables B.2 and B.3, respectively.

Figure B.1. Plasmid map of PV-ZMBK07.

Restriction sites, and their locations in base pairs, used during Southern blot analyses are shown.

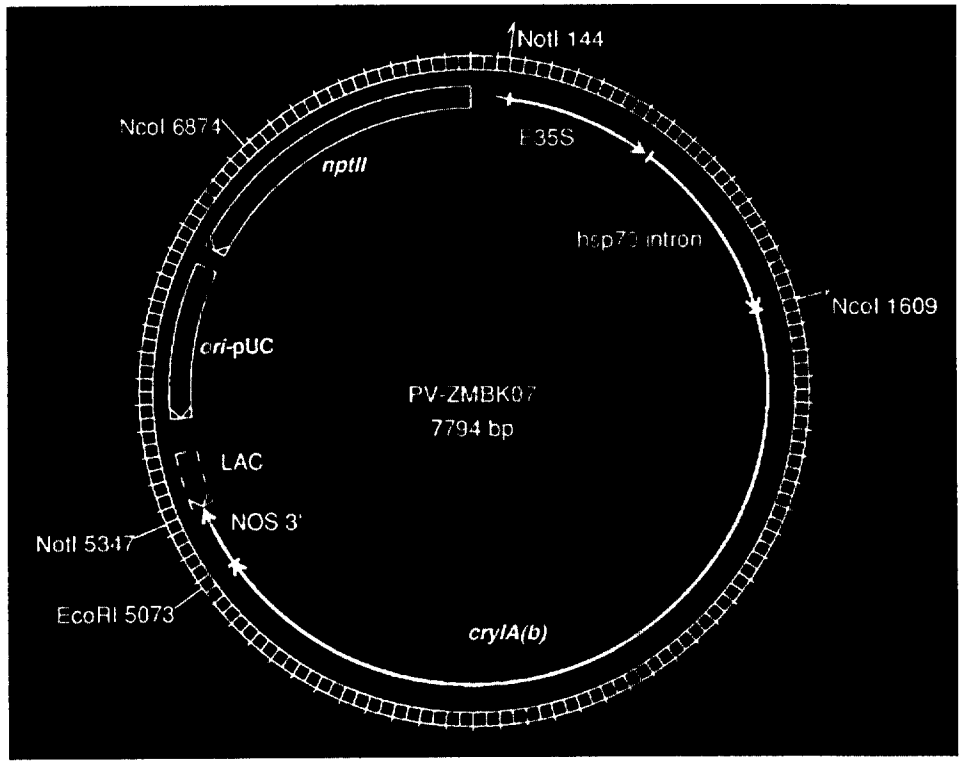


Table B.2 Summary of DNA Elements in the Plasmid PV-ZMBK07

Genetic Element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985*) with the duplicated enhancer region (Kay *et al.*, 1985*).
hsp70	0.80	Intron from the maize *hsp70* gene (heat-shock protein) present to intron increase the level of gene transcription (Rochester *et al.*, 1986*).
cryIA(b)	3.46	The gene encodes the nature identical CryIA(b) protein product (Fischhoff *et al.*, 1987*).
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983*).
lacZ	0.24	A partial *E. coli lacI* coding sequences, the promoter Plac, and a partial coding sequence for beta-D-galactosidase or *lacZ* protein from pUC119 (Yanisch-Perron *et al.*, 1985*).
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli* (Vieira and Messing, 1987*).
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid (Beck *et al.*, 1982*).

Figure B.2. Plasmid map of PV-ZMGT10.

Restriction sites, and their locations in base pairs, used during Southern analyses are shown.

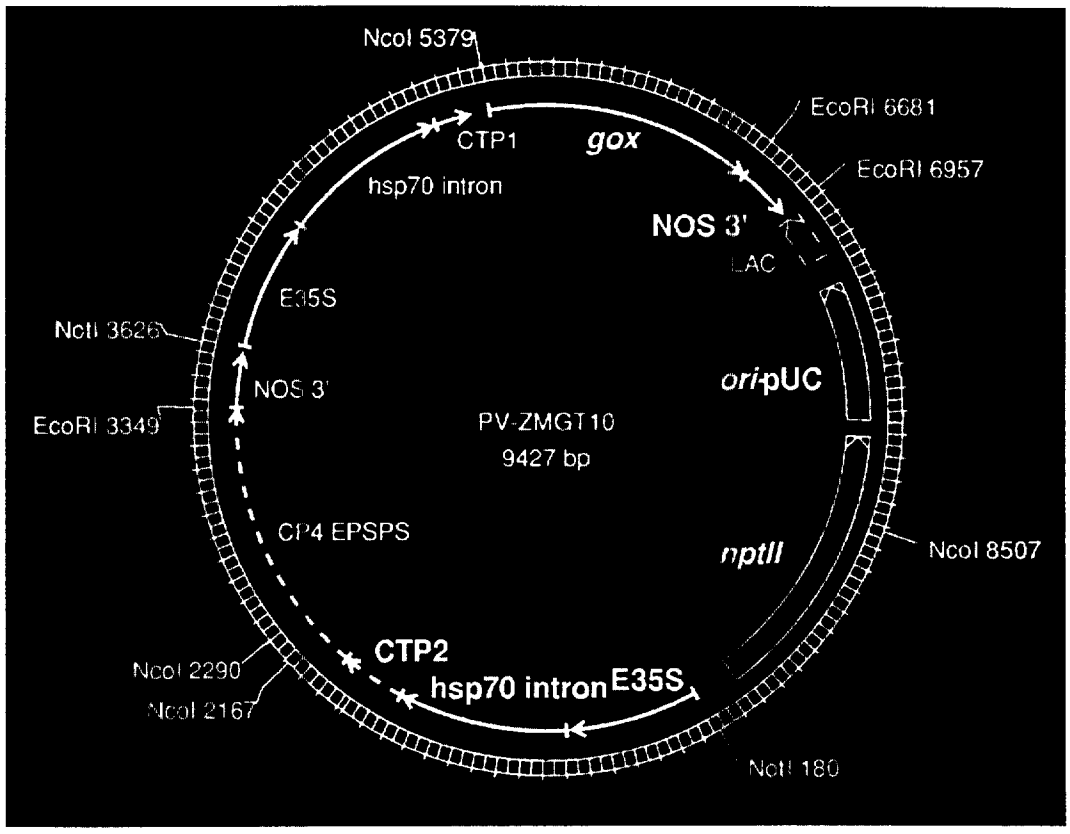


Table B.3 Summary of DNA Elements in the Plasmid PV-ZMGT10

Genetic Element	Size Kb	Function
E35S	0.61	The cauliflower mosaic virus (CaMV) promoter (Odell *et al.*, 1985*) with the duplicated enhancer region (Kay *et al.*, 1985*).
hsp70	0.80	Intron from the maize *hsp70* gene (heat-shock protein) present to intron increase the level of gene transcription (Rochester *et al.*, 1986*).
CTP2	0.31	Chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS (Klee and Rogers, 1987*), present to direct the CP4 EPSPS protein to the chloroplast, the site of aromatic amino acid synthesis.
CP4 EPSPS	1.4	The gene for CP4 EPSPS, isolated from *Agrobacterium* sp. strain CP4 (Harrison *et al.*, 1993a*) which allows for the selection of transformed cells on glyphosate.
CTP1	0.26	Chloroplast transit peptide, isolated from the small subunit gene of ribulose-1,5-bisphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana* (Timko *et al.*, 1988*), present to direct the GOX protein to the chloroplast, the site of aromatic amino acid synthesis.
gox	1.3	The gene encodes the glyphosate metabolizing enzyme glyphosate oxidoreductase (GOX), isolated from *Achromobacter* sp. (new genus *Ochrobactrum anthropi*) strain LBAA (Hallas *et al.*, 1988*; Barry *et al.*, 1992*; Barry *et al.*, 1994).
NOS 3'	0.26	A 3' nontranslated region of the nopaline synthase gene which terminates transcription and directs polyadenylation (Fraley *et al.*, 1983*).
lacZ	0.24	A partial *E. coli lacI* coding sequences, the promoter Plac, and a partial coding sequence for beta-D-galactosidase or *lacZ* protein from pUC119 (Yanisch-Perron *et al.*, 1985*).
ori-pUC	0.65	The origin of replication for the pUC plasmids that allows for plasmid replication in *E. coli* (Vieira and Messing, 1987*).
nptII	0.79	The gene for the enzyme neomycin phosphotransferase type II. This enzyme confers resistance to aminoglycoside antibiotics and thereby allows for selection of bacteria containing the plasmid (Beck *et al.*, 1982*).

(b) Description of the vector construction

Maize line MON 810 was produced by transforming the maize genotype Hi-II with a DNA solution containing two plasmid vectors, PV-ZMBK07 and PV-ZMGT10. The PV-ZMBK07 plasmid contains the *cryIA(b)* gene and PV-ZMGT10 contains the CP4 EPSPS and *gox* genes. Both plasmids contain the *nptII* gene under the control of a bacterial promoter and an origin of replication from a pUC plasmid, required for selection and replication of the plasmids in bacteria, respectively. A description of the DNA elements in PV-ZMBK07 and PV-ZMGT10 are given in Tables B.2 and B.3, respectively. Although the details of vector PV-ZMGT10 are provided, none of the DNA sequences from vector PV-ZMGT10 are present in Insect-Protected maize line MON 810 (see question 30(c)).

i. Plant expression vector - PV-ZMBK07

The plasmid vector PV-ZMBK07 (Figure B.1) contains the *cryIA(b)* gene under the control of the enhanced CaMV 35S promoter (E35S) (Kay *et al.*, 1985* and Odell *et al.*, 1985*), which is approximately 0.6 Kb in size. Located between the E35S promoter and the *cryIA(b)* gene is the 0.8 Kb intron from the maize *hsp70* gene (heat-shock protein), present to increase the levels of gene transcription (Rochester *et al.*, 1986*). The *hsp70* intron is followed by the 3.46 Kb *cryIA(b)* gene. The *cryIA(b)* gene is joined to the 0.26 Kb nopaline synthase 3' nontranslated sequence, NOS 3', (Fraley *et al.*, 1983*) which provides the mRNA polyadenylation signal.

The *cryIA(b)* gene is 3468 nucleotides in length and encodes a full-length *B.t.k.* HD-1 [CryIA(b)] protein of 1156 amino acids (Fischhoff *et al.*, 1987*), which when subjected to trypsin yields an active trypsin-resistant protein product of approximately 600 amino acids *in planta* and *in vitro* (Lee *et al.*, 1995b). The *cryIA(b)* gene sequence was modified to increase the levels of expression in maize (Perlak *et al.*, 1991*). The *cryIA(b)* gene encodes the nature identical CryIA(b) protein product (Fischhoff *et al.*, 1987*). The deduced amino acid sequence for the CryIA(b) protein is given in Figure B.3.

The *alpha* region of the *lacZ* gene for beta-galactosidase, present under a bacterial controlled promoter, is present in PV-ZMBK07. This region contained a polylinker (region of multiple cloning sites) which allowed for the cloning of the desired genes within the plasmid vector (Vieira and Messing, 1987*). The *lacZ-alpha* region is followed by the 0.65 Kb origin of replication for the pUC plasmids (*ori-pUC*) and which allows for the replication of plasmids in *E. coli* (Vieira and Messing, 1987*).

Following the *ori-pUC* region is the gene for the enzyme neomycin phosphotransferase type II (*nptII*). This enzyme confers resistance to aminoglycoside antibiotics (*i.e.*, kanamycin and neomycin) and was used for selection of bacteria during the construction of this plasmid. The coding sequence for the *nptII* gene was derived from the prokaryotic transposon Tn5 (Beck *et al.*, 1982*) and is present under its own bacterial promoter.

Figure B.3. Deduced amino acid sequence of the CryIA(b) protein as encoded by vector PV-ZMBK07.

```
   1  MDNNPNINEC IPYNCLSNPE VEVLGGERIE TGYTPIDISL SLTQFLLSEF
  51  VPGAGFVLGL VDIIWGIFGP SQWDAFLVQI EQLINQRIEE FARNQAISRL
 101  EGLSNLYQIY AESFREWEAD PTNPALREEM RIQFNDMNSA LTTAIPLFAV
 151  QNYQVPLLSV YVQAANLHLS VLRDVSVFGQ RWGFDAATIN SRYNDLTRLI
 201  GNYTDHAVRW YNTGLERVWG PDSRDWIRYN QFRRELTLTV LDIVSLFPNY
 251  DSRTYPIRTV SQLTREIYTN PVLENFDGSF RGSAQGIEGS IRSPHLMDIL
 301  NSITIYTDAH RGEYYWSGHQ IMASPVGFSG PEFTFPLYGT MGNAAPQQRI
 351  VAQLGQGVYR TLSSTLYRRP FNIGINNQQL SVLDGTEFAY GTSSNLPSAV
 401  YRKSGTVDSL DEIPPQNNNV PPRQGFSHRL SHVSMFRSGF SNSSVSIIRA
 451  PMFSWIHRSA EFNNIIPSSQ ITQIPLTKST NLGSGTSVVK GPGFTGGDIL
 501  RRTSPGQIST LRVNITAPLS QRYRVRIRYA STTNLQFHTS IDGRPINQGN
 551  FSATMSSGSN LQSGSFRTVG FTTPFNFSNG SSVFTLSAHV FNSGNEVYID
 601  RIEFVPAEVT FEAEYDLERA QKAVNELFTS SNQIGLKTDV TDYHIDQVSN
 651  LVECLSDEFC LDEKKELSEK VKHAKRLSDE RNLLQDPNFR GINRQLDRGW
 701  RGSTDITIQG GDDVFKENYV TLLGTFDECY PTYLYQKIDE SKLKAYTRYQ
 751  LRGYIEDSQD LEIYLIRYNA KHETVNVPGT GSLWPLSAPS PIGKCAHHSH
 801  HFSLDIDVGC TDLNEDLGVW VIFKIKTQDG HERLGNLEFL EGRAPLVGEA
 851  LARVKRAEKK WRDKREKLEW ETNIVYKEAK ESVDALFVNS QYDRLQADTN
 901  IAMIHAADKR VHSIREAYLP ELSVIPGVNA AIFEELEGRI FTAFSLYDAR
 951  NVIKNGDFNN GLSCWNVKGH VDVEEQNNHR SVLVVPEWEA EVSQEVRVCP
1001  GRGYILRVTA YKEGYGEGCV TIHEIENNTD ELKFSNCVEE EVYPNNTVTC
1051  NDYTATQEEY EGTYTSRNRG YDGAYESNSS VPADYASAYE EKAYTDGRRD
1101  NPCESNRGYG DYTPLPAGYV TKELEYFPET DKVWIEIGET EGTFIVDSVE
1151  LLLMEE
```

ii. Plant expression vector - PV-ZMGT10

This description of the functions of the PV-ZMGT10 sequence is purely informative, as none of the gene sequences of the PV-ZMGT10 vector have been detected in the Insect-Protected maize line MON 810.

The PV-ZMGT10 plasmid (Figure B.2) contains the *gox* and CP4 EPSPS genes joined to chloroplast transit peptides CTP1 and CTP2, respectively. Both coding regions are under the control of the enhanced CaMV 35S promoter, maize *hsp70* intron and NOS 3' terminator sequences. The PV-ZMGT10 vector contains the same *lacZ-alpha*, *ori-pUC* and *nptII* regions as described above for PV-ZMBK07.

The CP4 EPSPS gene was isolated from *Agrobacterium* sp. strain CP4 (Barry *et al.*, 1992*) and has been shown to have the potential to provide high resistance to glyphosate inhibition when introduced into plants (Padgette, *et al.*, 1993). Glyphosate binds to and blocks the activity of its target enzyme, EPSPS, an enzyme of the aromatic amino acid biosynthetic pathway. The CP4 EPSPS protein represents one of many different EPSPSs found in nature (Schulz *et al.*, 1985*). CP4 EPSPS is highly tolerant to inhibition by glyphosate and has high catalytic efficiency, compared to most EPSPSs (Barry *et al.*, 1992; Padgette *et al.*, 1993). Plant cells expressing the CP4 EPSPS protein are tolerant to glyphosate when present in growth medium since the continued action of the tolerant EPSPS enzyme meets the needs for aromatic compounds. The CP4 EPSPS gene is not contained within line MON 810 (see question 30 (c)).

The CP4 EPSPS gene in PV-ZMGT10 contains a chloroplast transit peptide, CTP2, isolated from *Arabidopsis thaliana* EPSPS (Klee and Rogers, 1987*) which targets the CP4 EPSPS protein to the chloroplast, the location of EPSPS in plants and the site of aromatic amino acid synthesis (Kishore and Shah, 1988*). The CP4 EPSPS gene with its CTP2 is approximately 1.7 Kb in size. The CP4 EPSPS gene cassette (promoter through 3' termination sequence) is joined to the *gox* cassette.

The *gox* gene that encodes the glyphosate metabolising enzyme glyphosate oxidoreductase (GOX) was cloned from *Achromobacter* sp. (new genus *Ochrobactrum anthropi*) strain LBAA, (Hallas *et al.*, 1988*; Barry *et al.*, 1992*; Barry *et al.*, 1994). The GOX protein is targeted to the plastids with a chloroplast transit peptide sequence, CTP1. The CTP1 was derived from the small subunit gene of ribulose-1,5-bisphosphate carboxylase (SSU1A) gene from *Arabidopsis thaliana* (Timko *et al.*, 1988*). The enzyme GOX degrades glyphosate by converting glyphosate to aminomethylphosphonic acid (AMPA) and glyoxylate (Padgette *et al.*, 1994). The *gox* gene is not in line MON 810 (see question 30 (c)).

(c) Genetic map and/or restriction map of the vector

The plasmid map of vector PV-ZMBK07 is shown in Figure B.1 and PV-ZMGT10 is shown in Figure B.2. Restriction sites are noted on each map.

(d) Sequence data

The DNA sequence of plasmid PV-ZMBK07 is Confidential Business Information and is given in Appendix II.

(e) Information on the degree to which the vector contains sequences whose product or function area is not known

All sequences within plasmid vectors PV-ZMBK07 and PV-ZMGT10 are completely known including product and function area.

(f) Genetic transfer capabilities of the vector

Not applicable.

(g) Frequency of mobilization of the vector

Not applicable.

(h) Part of the vector which remains in the GMO

Portions of the same or different plasmids can become fragmented and rejoined (i.e. rearranged) during the particle acceleration transformation process (Koziel *et al.*, 1993; Padgette *et al.*, 1995*; Register *et al.*, 1994; Spencer *et al.*, 1992*; Vasil *et al.*, 1992*; Wan *et al.*, 1995*). In maize line MON 810, a less than full length *cryIA(b)* gene has been incorporated (Section 30(c)). The CryIA(b) protein is expressed at levels which provide protection from certain lepidopteran insects including European corn borer. Molecular analysis has established that only the *cryIA(b)* gene was integrated into the genome; there was no evidence of the CP4 EPSPS, *gox* and *nptII* genes and the *ori-pUC* (Kania *et al.*, 1995 - Appendix I). The GOX and CP4 EPSPS proteins were not detected in Insect-Protected line MON 810. For more information on the portion of the plasmids that were inserted into the maize genome see question 30(c) below.

30. Information on the insert

(a) Methods used to construct the insert

See question 29.

(b) Restriction sites

See question 29.

(c) Sequence of the insert

i. Molecular Analysis of Insect-Protected Maize Line MON 810

The integrated DNA in Insect-Protected maize line MON 810 was analysed for insert number (number of integration sites within the maize genome) and the number of copies of each gene (Kania, *et al.*, 1995, Appendix I).

Maize line MON 810 was produced by particle acceleration technology with a DNA solution containing two plasmids: PV-ZMBK07 [which contains the *cryIA(b)* gene] and PV-ZMGT10 [which contains the CP4 EPSPS and *gox* genes]. No sequences from plasmid PV-ZMGT10 were integrated into maize line MON 810. This line contains one integrated DNA contained on an approximately 5.5Kb NdeI fragment, which contains a single copy of the E35S promoter, the *hsp*70 intron and the *cryIA(b)* gene. The *nptII* gene and backbone sequences of plasmid PV-ZMBK07 were not integrated. This line does not contain the CP4 EPSPS, *gox* or *nptII* genes, nor the plasmid backbone from plasmid PV-ZMGT10.

Table B.4 Summary of Maize Line MON 810 Molecular Analysis

Genetic Element	Maize Line MON 810
cryIA(b) gene	1 copy present
CP4 EPSPS gene	not present
gox gene	not present
*nptII/ori-p*UC	not present

(d) Origin and function of each constituent part of the insert in the GMO

The nucleotide sequences inserted in maize line MON 810 contain the *cryIA(b)* gene (origin: *Bacillus thuringiensis* subsp. *kurstaki,* strain HD-1), under the control of an enhanced CaMV 35S promoter (origin: cauliflower mosaic virus) and an intron from the heat shock protein 70 gene (*hsp*70) (origin: maize) located between the enhanced CaMV 35S (E35S) promoter and the *cryIA(b)* gene.

The *cryIA(b)* gene has been introduced into Insect-Protected maize line MON 810 to provide control of certain lepidopteran insects including European corn borer (*Ostrinia nubilalis*) and the pink borer (*Sesamia cretica*). The E35S promoter directs the expression of the CryIA(b) protein in the target tissues (Odell *et al.*, 1985*). The *hsp*70 intron increases the expression of the CryIA(b) protein (Rochester *et al.*, 1986*).

(e) Information on the degree to which the insert is limited to the required function

The only DNA sequence inserted into maize line MON 810 are those required for the expression of the *cryIA(b)* gene which confers the insect-protected phenotype.

(f) Location of the insert in the GMO

The insert is stably integrated into the plant chromosome and is inherited as a single dominant gene in Mendelian fashion. This has been confirmed by inheritance patterns and by molecular analysis (see question 37).

INFORMATION ON THE ORGANISM(S) FROM WHICH THE INSERT IS DERIVED (DONOR)

31. Scientific and other names

The inserted DNA sequences were derived from the following donor organisms:
Cauliflower mosaic virus (CaMV) (virus) - enhanced 35S promoter (E35S)
Maize (plant) - *hsp 70* intron
Bacillus thuringiensis (bacterium) - *cryIA(b)* gene

32(a) Pathogenic characteristics of the donor organism

Detailed information concerning the donor organisms is not considered relevant to the risk assessment of the Insect-Protected maize plants, since transformation, regeneration, and molecular analysis techniques have established that only specific isolated nucleotide sequences have been transferred to the maize plants.

32(b) Other harmful characteristics of the organism living or dead, including its extracellular products

See question 32(a).

33. If the donor organism has any pathogenic or harmful characteristics, indicate whether the donated sequences are in any way involved in them

None of the inserted sequences are known to have any pathogenic or harmful characteristics.

34. Classification under existing Community rules relating to the protection of human health and the environment

Not relevant.

35. Potential for natural exchange of genetic material between the donor(s) and recipient organism

The recipient and donor organisms are not related with exception of the *hsp70* intron which was isolated from maize.

INFORMATION RELATING TO THE GMO(S) CONTAINED IN THE PRODUCT

36. Description of genetic traits or phenotypic characteristics and in particular any new traits and characteristics which may be expressed or no longer expressed

Insect-Protected maize plants provide effective control of European corn borer (*Ostrinia nubilalis*) and certain other lepidopteran pests in maize such as pink borer (*Sesamia cretica*) (two economically damaging insects). These genetically modified maize plants produce the CryIA(b) insect control protein derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). European corn borer (ECB) damage to maize plants includes: (1) leaf feeding (from the first insect generation), (2) stalk tunnelling (from the first and second generations), (3) leaf sheath and collar feeding (from the second and third generations), and (4) ear damage (from the second and third generations). Current chemical insecticides for control of ECB offer limited utility as applications must be made prior to the time the insect bores into the stalk and repeat applications are often necessary.

The use of Insect-Protected maize would enable the farmer to effectively control ECB providing protection of potential maize yield and a reduction of the use of chemical insecticides for this insect pest. Insect-Protected maize would provide benefits to growers, the general public, and the environment, including: (1) a more reliable, economical, and less labour intensive means to control ECB, (2) insect control without harming nontarget species, (3) a means for growers to significantly reduce the amount of chemical insecticides now applied to the crop thereby achieving ECB control in a more environmentally compatible manner than is currently available, (4) a reduction in the manufacturing, shipment, and storage of chemical insecticides used in maize, (5) a reduction in the exposure to workers to the pesticide and pesticide spray solution, (6) a reduction in the number of empty pesticide containers and amount of spray solution that must be

disposed of according to applicable environmental regulations, (7) a fit with integrated pest management (IPM) and sustainable agricultural systems, and (8) both large and small growers will benefit from the planting of Insect-Protected maize as no additional labour, planning, or machinery is required.

37. Genetic stability of the GMO

The inserted *cryIA(b)* gene has been shown to be stably integrated into the plant chromosome based on segregation data and Southern analysis.

(a) Segregation data and stability of gene transfer in Insect-Protected maize line MON 810

Segregation data for the BC0F1 plants (derived from crossing the R0 with an inbred line), BC1F1 plants (derived from crossing the BC0F1 plants to the same inbred used to cross with the R0 plant), and BC1F2 progeny (derived from crossing individual BC0F2 plants by a non-transgenic tester and analysing subsequent generation ear to row) are presented in Table B.5. The results are consistent with a single active insert segregating according to Mendelian genetics.

Table B.5 Segregation Data and Analysis of Progeny of Insect-Protected Maize Line MON 810

Generation	Actual	Expected	ChiSq
BC0F1[1]	44:47	45.5:45.5	0.044 *
BC1F1[2]	10:4	7:7	1.786 *
BC1F2 progeny [3]	69:181:77	81.75:163.5:81.75	4.138 #

[1] Data expressed as number of expressing plants: number of non-expressing plants based on European corn borer feeding assay

[2] Data expressed as number of expressing plants. number of non-expressing plants based on CryIA(b) ELISA

[3] Data expressed as number of ear rows with homozygous expressing plants. number of ear rows with segregating plants number of ear rows with homozygous susceptible plant based on European corn borer feeding assay

* not significant at p = 0 05 (chi square = 3.84, 1 df)

\# not significant at p = 0 05 (chi square = 5.99, 2 df)

The *cryIA(b)* gene in Insect-Protected maize line MON 810 has been shown to be stable through seven generations of crosses to one recurrent parent (B73) and six generations of crosses to a second, unrelated inbred (Mo17) (Table B.6.). The Chi square tests for the backcross to B73 and to Mo17 did not deviate from expectations at p=0.05.

Table B.6: Stability of Gene Transfer Based on Segregation Data for Backcross Derivatives of Insect-Protected Maize Line MON 810 in Two Unrelated Inbred Lines (B73 and Mo17). Values are ratios of plants that are positive or negative for the CryIA(b) protein as determined by ELISA.

Generation	Actual	Expected	ChiSq
BC6F1(B73) [1]	8:13	10.5:10.5	0.762 *
BC5F1(Mo17) [1]	11:11	11:11	0.045 *

[1] Data expressed as number of expressing plants: number of non-expressing plants based on *B.t.k.* CryIA(b) ELISA

* not significant at p = 0.05 (chi square = 3.84, 1 df)

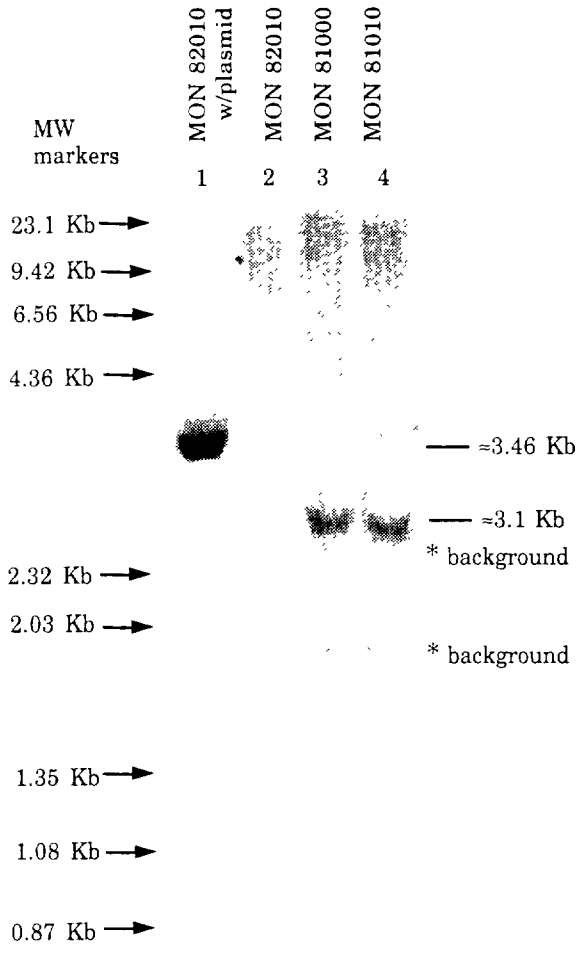
To summarise the segregation and stability data (Tables B.5 and B.6), the data are consistent with a single active site of insertion of the *cryIA(b)* gene into genomic DNA of line MON 810. The stability of this insertion has been demonstrated through seven generations of crossing. Generation of homozygous lines (Table B.6) further demonstrates the stability of the insert.

(b) Insect-Protected Maize Line MON 810 Generation Stability: Southern Blot Analysis

Insect-Protected maize line MON 810 has been planted for two years in field trials in the United States. Seed used to plant these trials represents various stages in the breeding program. Leaf tissue samples from two sets of MON 810 plants, representing three generations, were used to assess the stability of the inserted DNA by Southern blot analysis (Southern, 1975). Maize line MON 810 planted in the 1994 field trials, seed batch MON 81000 (lane 3), represents the F1 generation of breeding with B73. The maize line MON 810 planted in the 1995 field trials, seed batch MON 81010 (lane 4), was a hybrid derived from additional breeding (F4). The non-transgenic control line, MON 820 (seed batch MON 82010), was run spiked with the transformation plasmids (lane 1, as size marker and hybridization control) and alone (lane 2) as controls. The maize DNAs were digested with the restriction enzymes NcoI/EcoRI and probed with a portion of the *cryIA(b)* gene.

The MON 820 DNA plus plasmids (lane 1) shows the *cryIA(b)* fragment, 3.46 Kb as present in the transformation plasmid. The control DNA alone, MON 820 (lane 2) shows two light background bands due to non-specific hybridization (indicated with asterisks). Both MON 810 DNAs (lanes 3 and 4), representing different breeding lineages, contain the same 3.1 Kb NcoI/EcoRI *cryIA(b)* fragment (Kania *et al.*, 1995). This Southern blot analysis demonstrates that the insertion event has been stable during maize breeding. The continued efficacy of this line in controlling ECB during breeding also supports the molecular stability of the inserted DNA.

Figure B.4: **Southern blot analysis of genetic stability of Insect-Protected maize line MON 810**



Southern blot analysis of insect protected maize line MON 810 DNA. Lanes 1-4 contain the following DNAs digested with NcoI/EcoRI and probed with the cryIA(b) gene: lane 1, MON 82010 DNA with ≈15 pg of PV-ZMBK07 and PV-ZMGT10; lane 2, MON 82010 DNA; lane 3, MON 81000 DNA; lane 4, MON 81010 DNA.

—► Symbol denotes sizes obtained from MW markers.
—≈ Symbol denotes a band size approximated from MW marker and plasmid digests.
* Symbol denotes background bands.

38. Rate and level of expression of the new genetic material

Using the CaMV 35S promoter, a constitutive promoter, it is expected that the CryIA(b) protein encoded by the *cryIA(b)* gene under the control of the CaMV 35S promoter will be expressed in most tissues of the whole plant. This was confirmed by ELISA analyses.

Tissues of MON 810 plants were analysed for the three proteins, CryIA(b), CP4 EPSPS, and GOX. The CP4 EPSPS and GOX proteins were not detected in any of the plant tissues of maize line MON 810. This was expected since the molecular analysis of maize line MON 810 established that the CP4 EPSPS and *gox* genes were not present in the integrated DNA (see question 30(c) and Kania *et al.*, 1995 in Appendix I). Expression of the CryIA(b) protein in Insect-Protected maize line MON 810 is constitutive, the CryIA(b) protein was detected in all tissues analysed (Sanders *et al.*, 1995). This was expected from use of the CaMV 35S promoter (Odell *et al.*, 1985*).

(a) Expression Levels in Tissues of Line MON 810 from the 1994 US Field Trials

In 1994, field trials were conducted at six locations distributed throughout the major U.S. maize growing region representing a variety of environmental conditions (Sanders *et al.*, 1995). Plant samples from MON 810 and the control, MON 818, were collected from each site. CryIA(b), CP4 EPSPS, and GOX protein levels were assessed in maize tissues using validated ELISAs specific for each protein (Table B.7) (Ledesma *et al.*, 1995; Davies and Sanders, 1995; Elswick 1995). The whole plant was collected two weeks following pollination. Young leaves were collected three times at two week intervals for estimating foliar expression levels during the growing season (overseason leaf expression). Since the *nptII* gene is under the control of a bacterial-specific promoter and lack of expression was previously demonstrated for Insect-Protected maize line MON 801 in 1993 (Davies 1995), further NPTII analysis was not performed in 1994.

Table B.7 Summary of Specific Protein Levels in Tissues of Maize Line MON 810 from the 1994 US Field Trials[1]

		Protein (µg / g fwt)		
		CryIA(b)	CP4 EPSPS[2]	GOX[2]
Leaf	mean[3]	9.35	N D [4]	N D
	range[5]	7.93-10.34	N A.[6]	N A
Whole Plant[7]	mean	4.15	N D	N D.
	range	3.65-4.65	N A.	N.A.
Grain	mean[3]	0.31	N D	N D.
	range[5]	0.19-0.39	N A.	N.A.

Overseason Leaf[8] - CryIA(b) protein	1st	2nd	3rd
mean	9.78	8.43	4.91

[1] There were six field sites. All values are expressed as µg / g fresh weight of tissue.

[2] Molecular analysis established that the CP4 EPSPS and gox genes are not present in maize line MON 810.

[3] The means were calculated from the analysis of one plant sample of pooled tissue from several plants per site unless noted otherwise.

[4] Not detected.

[5] The range is the minimum and maximum values from the analysis of samples from six sites.

[6] Not applicable; protein was not detected therefore there was no range of values

[7] The mean and range was calculated from the analysis of two plants collected from a single site. The whole plants (including the roots) were harvested two weeks following pollination

[8] The youngest leaves were collected at two week intervals during the growing season from one site.

The level of CryIA(b) protein ranged from 7.93-10.34 µg / g fwt in young leaf tissue, 3.65-4.65 µg / g fwt in whole plant tissue, and 0.19-0.39 µg / g fwt in harvested grain. The foliar expression of the CryIA(b) protein remained high during the vegetative growth stages of the maize plant as measured in overseason leaf samples.

The CP4 EPSPS and GOX proteins were not detected in any of the plant tissues of maize line MON 810.

(b) Expression Levels in Tissues of MON 810 from the 1995 European Field Trials

In 1995, five field trials were conducted within the major maize growing regions of France and Italy. The locations (Segoufielle, FR; Beaumont sur Lèze, FR; Le Castera, FR; Montadet, FR and Mogliano Veneto TV, IT) encompass a range of environmental conditions and insect pressure from agronomically important pests. Plant samples from MON 810 and the control, MON 820, were collected. Young leaf samples were collected from all sites; forage and grain samples were collected from all sites except Mogliano Veneto TV, which was destroyed prematurely. In the 1995 trial design, 'forage' plants were collected rather than 'whole plants' since forage plants are used as feed. The forage was harvested at soft dough stage. The CryIA(b), CP4 EPSPS, and GOX protein levels were assessed in maize samples using validated ELISAs specific for each protein (Table B.8).

Table B.8 Summary of Specific Protein Levels in Tissues of Maize Line MON 810 from the 1995 European Field Trials[1]

		Protein (µg / g fwt)		
		CryIA(b)	CP4 EPSPS[2]	GOX[2]
Leaf	mean[3]	8.60	N.D.[4]	N.D.
	range[5]	7.59-9.39	N.A.[6]	N.A.
Forage[7]	mean	6.08	N.D.	N.D.
	range	4.21-9.23	N.A.	N.A.
Grain[8]	mean	0.53	N.D.	N.D.
	range	0.42-0.69	N.A.	N.A.

1 There were five field sites planted. All values are expressed as µg / g fresh weight of tissue.

2 Molecular analysis demonstrated that the CP4 EPSPS and gox genes are not present in maize line MON 810

3 The means were calculated from the analysis of a single pooled sample from each site.

4 Not detected.

5 The range is the minimum and maximum values from the analysis of samples from five sites.

6 Not applicable, protein was not detected therefore there was no range of values.

7 The mean and range were calculated from the analysis of two pooled plants collected from 4 sites

8 The mean and range were calculated from the analysis of pooled ears collected from 4 sites

The level of CryIA(b) protein ranged from 7.59-9.39 µg / g fwt in young leaf tissue, 4.21-9.23 µg / g fwt in forage tissue, and 0.42-0.69 µg / g fwt in harvested grain. The CP4 EPSPS and GOX proteins were not detected in the plant tissues of maize line MON 810. The CryIA(b) protein levels in these tissues are similar for plants grown in the United States and European field trials over two consecutive generations.

(c) Expression Levels in the Tissues of Progeny from MON 810 from the 1995 European Field Trials

Field trials were conducted in both Italy and France to produce leaf, forage and grain samples for expression analysis of Insect-Protected maize hybrids. The five Insect-Protected maize hybrids were developed through crossing of the MON 810 event into commercial inbreds. Non-modified versions of the same hybrids were used as the controls. Leaf samples were collected at the Italy site only, while forage and grain samples were collected at both sites. The CryIA(b) protein levels were assessed in the maize samples using a validated ELISA (Table B.9). The ELISAs for CP4 EPSPS and GOX proteins were not performed since the genes are not present in maize line MON 810 (the absence of these proteins was confirmed in the previous field trials (Tables B.7. and B.8.). Field trials were approved under permit numbers B/IT/95-23 and 95.03.06 for Italy and France, respectively.

Table B.9. Summary of CryIA(b) Protein Levels in Tissues of Progeny from Maize Line MON 810 Grown in the 1995 E.U. Field Trials[1]

	CryIA(b) Protein (µg / g fwt)	
Leaf	mean[2]	9.26
	range[3]	8.20-10.51
Forage[4]	mean	4.52
	range	4.00-5.11
Grain[5]	mean	0.46
	range	0.35-0.60

[1] There were five hybrids planted at two field sites. All values are expressed as µg / g fresh weight of tissue.

[2] The means were calculated from the analysis of an aliquot of pooled sample from Italy site.

[3] The range is the minimum and maximum values from the analysis of samples from Italy site.

[4] The mean and range were calculated from the analysis of one or two plants collected from both sites.

[5] The mean and range were calculated from the analysis of pooled grain samples collected from both sites.

The level of CryIA(b) protein in progeny of MON 810 ranged from 8.20-10.51 µg / g fwt in young leaf tissue, 4.00-5.11 µg / g fwt in forage tissue, and 0.35-0.60 µg / g fwt in harvested grain. The CryIA(b) protein levels are similar for MON 810 plants derived from backcrosses to B73/Mo17 (Table B 7 and B.8.) and commercial hybrids (Table B 9.).

In summary, the level of CryIA(b) protein in MON 810 plants is similar when plants are grown in different geographies and when the gene is present in different genetic backgrounds. The level of expression remains consistently high to provide season long control of the targeted insect pests.

39. Activity of the expressed proteins

The CryIA(b) Protein

The CryIA(b) protein must be ingested by the insect to have an insecticidal effect (Huber and Lüthy, 1981*). The protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH (Bulla *et al.*, 1977*); however, the pH of the larval insect gut is alkaline which favours solubilisation of the protein crystal. The solubilised protein is subsequently activated by proteases in the insect gut. The activated protein, which consists of approximately 600 amino acids, diffuses through the peritrophic membrane of the insect to the midgut epithelium, binding to the specific high affinity receptors on the surface of the midgut epithelium of target insects (Wolfersberger *et al.*, 1986*; Hofmann *et al.*, 1988a*). The gut becomes paralysed as a consequence of changes in electrolytes and pH in the gut causing the larval insect to quit feeding and die.

The insecticidal activity of the CryIA(b) protein is specific to lepidopteran insects. Only seven of the eighteen insects screened were sensitive to *B. thuringiensis* subsp. *kurstaki* proteins (MacIntosh *et al.*, 1990*) and all were lepidopterans. This specificity is directly attributable to the presence of receptors in the target insects (Van Rie *et al.*, 1989*; Hofmann *et al.*, 1988a*).

There are no receptors for the protein delta-endotoxins of *Bacillus thuringiensis* subspecies on the surface of mammalian intestinal cells, therefore, humans are not susceptible to these proteins (Hofmann *et al.*, 1988b*; Noteborn and Kuiper, 1995*; Sacchi *et al.*, 1986*). In addition to the lack of receptors for the CryIA(b) proteins, the absence of adverse effects in humans is further supported by numerous reviews on the safety of the *B.t.* proteins (Ignoffo, 1973*; Shadduck, 1983*; Siegel and Shadduck, 1989*). Rodent feeding [redacted] and *in vitro* digestive fate studies of the CryIA(b) protein (Ream, 1994) confirm this conclusion See Section C.2 for a more complete discussion of this subject.

40(a) Description of detection techniques for the GMO in the environment

Southern blot or PCR techniques can be employed for the detection and identification of the inserted nucleotide sequences and ELISA for detection of the expressed CryIA(b) protein These techniques can be utilized to identify Insect-Protected maize plants expressing the *cryIA(b)* gene.

Additionally, an insect bioassay employing sensitive lepidopteran insect species such as tobacco hornworm (*Manduca sexta*), cabbage looper (*Trichoplusia ni*) or European corn borer, may be utilised to identify plants expressing the CryIA(b) protein (MacIntosh *et al.*, 1990*).

40(b) Description of identification techniques

Grain from Insect-Protected maize line MON 810 is substantially equivalent to grain from other maize varieties (See section C.2.). Molecular tools, such as Southern blots and PCR as described in this document (question 30) could be used to identify grain and seed from Insect-Protected maize. See question 40(a).

41. Health considerations

(a) toxic or allergenic effects of the non-viable GMOs and/or their metabolic products

Insect-Protected maize line MON 810 is protected against certain lepidopteran insects, due to the specificity of the CryIA(b) protein. The specificity of the CryIA(b) protein and its potential impacts on human health are discussed in detail in Sections C.1 and C.2. Based on this information, it is concluded that Insect-Protected maize line MON 810 is compositionally equivalent to other commercial maize varieties and the CryIA(b) protein poses no toxic or allergenic concerns.

(b) product hazards

No specific hazards have been identified in Insect-Protected maize line MON 810 or its progeny as a result of expressing the CryIA(b) protein.

(c) comparison of the GMO with the donor, recipient or parental organism regarding pathogenicity

Neither the host plant, maize, nor the donor organism of the introduced protein, are known to be pathogenic or harmful. Insect-Protected line MON 810 has been shown to be equivalent to other maize varieties, apart from the control of European corn borer and pink borer.

(d) capacity for colonization

Not relevant.

(e) If the organism is pathogenic to humans who are immuno-competent, supply the information specified in Annex II, Part II C 2 (i) (v)

Not relevant.

INTERACTIONS OF THE GMO WITH THE ENVIRONMENT

42. Survival, multiplication and dissemination of the GMO(s) in the environment

Insect-Protected maize line MON 810 and its progeny have been field tested in eighteen test sites in Europe since 1994 and over sixty test sites in the United States since 1993. Data collected from some of these trials (including yield, agronomic characteristics, vigour, disease, and insect susceptibility) demonstrate that this maize line has not been altered in survival, multiplication or dissemination characteristics when compared to other maize varieties.

On the basis of observations made in all of the test environments, there have been no phenotypic differences, except for the lepidopteran insect-protection of maize line MON 810, when compared to controls or other maize varieties. This conclusion is supported by data demonstrating that the grain of maize line MON 810 is substantially equivalent to the grain of other maize varieties (Section C.2).

(a) Survivability

i. germination rate and seed dormancy

Germination tests of Insect-Protected maize line MON 810 and control were conducted at five field locations in 1994 across the midwestern U.S. Results of these tests showed that all seed samples demonstrated high rates of germination and no differences were observed between lines MON 810 and the control under a range of environmental conditions (Table B.10). These findings support the conclusion that there are no differences in germination or dormancy between Insect-Protected maize line MON 810 and the control.

Table B.10. Field Germination Results for Insect-Protected Maize Line MON 810 and Control

Line	Percent germination	Range
MON 810[a]	87.4%	71.1 - 94.3 %
Control[a]	90.6%	78.9 - 98.3 %

[a] mean and range based upon five sites

ii. vegetative vigour

Based on documented visual observations during the field testing of Insect-Protected maize line MON 810 since 1993, no differences in vegetative vigour have been observed between line MON 810 and the parental control or other

maize lines of a similar genetic background. No differences in agronomic quality, disease, or insect susceptibility other than insect-protection were detected between line MON 810 and non-genetically modified plants (Croon *et al.*, 1995). Diseases monitored included northern leaf blight (*Exserohilum turcicum),* southern leaf blight (*Bipolaris maydis*), bacterial leaf blight (*Erwinia stewartii*), common maize smut (*Ustilago maydis*), maize stripe virus and common maize rust (*Puccinia sorghi*).

Observations since 1993 demonstrate that there are no differences in vegetative vigour or adaptation to environmental stress factors including drought, heat and frost, between Insect-Protected maize line MON 810 and the parental control or other maize lines of similar genetic background.

(b) Modes and/or rate of reproduction

Extensive observations recorded by field cooperators in 1993, 1994, and 1995 field trials demonstrate that the mode and rate of reproduction of Insect-Protected maize line MON 810 are typical of other maize varieties (Croon *et al.*, 1995). This maize line continues to exhibit the same separate staminate (tassel) and pistillate (silk) features. Pollen was produced entirely in the staminate inflorescence with anthesis (pollen shed) synchronous with silk emergence. No differences in seed or plant maturity have been observed. In some trials, non-insect protected plants matured more rapidly due to premature senescence caused by stalk boring insect damage.

No difference in yield between Insect-Protected maize line MON 810 and the control line protected by standard commercial insecticide products was observed in 1994 and 1995 yield trials.

(c) Dissemination

Dissemination of maize plants occurs exclusively through the seed. The introduced traits may also be dispersed by pollen. Maize cannot survive without human assistance due to past selection in its evolution (Galinat, 1988). Consequently, seed dispersal of individual kernels naturally does not occur because of the structure of the ears of maize. The introduced trait, insect-protection, had no influence on reproductive morphology and hence no changes in seed dissemination would be expected. Insect-Protected maize line MON 810 continued to show a polystichous female inflorescence (ear) on a stiff central spike (cob) enclosed in husks (modified leaves).

No trials have been conducted to specifically study pollen production or pollen dispersal. However, based on the fact that pollen production and pollen viability (as measured by yield and germination (viability of the embryo) of progeny) are unchanged by the genetic modification, the outcrossing frequency is unlikely to be different for Insect-Protected maize line MON 810 when compared to other varieties.

43. Interactions of the GMOs with the environment

The behaviour and characteristics of Insect-Protected maize plants have been studied in a range of field environments since 1992 in the U.S. and no significant differences, compared to other maize varieties, have been observed, apart from protection from certain lepidopteran insects. The assessment of the interactions with the environment have included:

- susceptibility to insects and diseases
- survival capacity (volunteers)
- seed multiplication capacity (yields)
- CryIA(b) protein expression in leaves and grain
- seed composition analysis
- safety for birds
- safety for mammals

In all of the environments where Insect-Protected maize line MON 810 has been tested, no differences in behaviour have been observed between this line and other maize varieties. See also question 42 and section C.1.

44. Environmental impacts of the GMO(s)

The environmental impact of the grain of Insect-Protected maize is not expected to be any different from that of other maize varieties used for the same purposes (see questions 42 and 43 and section C.1).

The production of Insect-Protected maize will provide benefits to farmers, the general public and the environment, including: 1) a more reliable and economical means to control ECB; 2) insect control without harming non-target species and benefical insects; 3) reduction in the use of chemical insecticides to control ECB; 4) a reduction in the manufacturing, shipment, and storage of chemical insecticides; 5) a reduction in worker exposure to pesticides; 6) a fit with Integrated Pest Management (IPM) and sustainable agricultural systems; and 7) both large and small growers will benefit from the planting of Insect-Protected maize as no additional labour, planning or machinery is required.

The risk of resistance of European corn borer populations to the CryIA(b) protein has been taken into consideration by Monsanto in order to maintain the sustainability of the Insect-Protected Maize. Monsanto has developed strategies to address and manage the risk of resistance. The various elements of this approach are discussed in section C-1-e and in Appendix III.

C. PREDICTED BEHAVIOUR OF THE PRODUCT

1. Environmental impact of the product

No adverse effects on the environment are expected from the production or import of Insect-Protected maize seed, grain or its derived products (see questions B.42 to 44). Further, there is extensive information on the lack of non-target effects from microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) containing the CryIA(b) protein. The full length CryIA(b) protein encoded by the *cryIA(b)* gene used to produce these Insect-Protected maize plants and the insecticidally active trypsin-resistant core protein produced in these plants are identical to the respective full length and trypsin-resistant core CryIA(b) proteins contained in microbial formulations that have been used safely commercially for over 30 years (Lee *et al.*, 1995a; EPA, 1988*). *B.t.k.* proteins are extremely selective for the lepidopteran insects (MacIntosh *et al.*, 1990*; Klausner, 1984*; Aronson *et al.*, 1986; Dulmage, 1981*; Whitely and Schnepf, 1986*), bind specifically to receptors on the mid-gut of lepidopteran insects (Wolfersberger *et al.*, 1986*; Hofmann *et al.*, 1988a*; Hofmann *et al.*, 1988b*; Van Rie *et al.*, 1989*; Van Rie *et al*, 1990*) and have no deleterious effect on beneficial/non-target insects, including predators and parasitoids of lepidopteran insect pests or honeybee (*Apis mellifera*) (Flexner *et al.*, 1986*; Krieg and Langenbruch, 1981*; Cantwell *et al.*, 1972*; EPA, 1988*; Vinson, 1989*; Melin and Cozzi, 1989*). The safety of the CryIA(b) protein and the maize plants expressing this protein to non-target insects was confirmed in field trials conducted in 1995 in France and Italy (Appendix IV).

In addition, separate studies were undertaken to assess the safety of the CryIA(b) protein to other non-target insects. These studies were conducted with the trypsin-resistant core of the CryIA(b) protein since this is the insecticidally active portion of the CryIA(b) protein and the insecticidally active form of the CryIA(b) protein detected in Insect-Protected maize line MON 810 (Lee *et al.*, 1995b). The chemical and functional equivalence of the trypsin-resistant core of the CryIA(b) protein produced in *E. coli* and in Insect-Protected maize plants, including line MON 810, was established using a rigorous set of criteria including molecular weight, immunoreactivity and insecticidal activity (Lee *et al.*, 1995b).

(a) Honey bee larvae and adults

These studies were performed to assess the safety of the CryIA(b) trypsin-resistant core protein for larvae and adult honey bee (*Apis mellifera* L.), a beneficial insect pollinator. A maximum hazard dose was used for these studies. The maximum nominal CryIA(b) protein concentration tested was greater than 10 times the estimated level required for 50% mortality (LC_{50}) of several target pest Lepidoptera to the CryIA(b) protein (MacIntosh *et al*, 1990*). No differences among treatments were observed and the LC_{50} for CryIA(b) protein in larval and adult honey bee was greater than 20 ppm, the highest dose tested. The no observed effect level was 20 ppm (Maggi and Sims, 1994a and 1994b; Sims, 1994).

(b) Green lacewing

A study was performed to assess the safety of the CryIA(b) trypsin-resistant core protein for green lacewing larvae (*Chrysopa carnea*), a beneficial predatory insect commonly found in maize and other cultivated plants. There was no evidence that green lacewing larvae were adversely effected when fed moth eggs coated with a nominal concentration of 16.7 ppm CryIA(b) protein for seven days. Under the conditions of the test, the LC_{50} was greater than 16.7 ppm CryIA(b) protein, the highest dose tested (Hoxter and Lynn, 1992a).

(c) Parasitic hymenoptera

This study was performed to assess the safety of the CryIA(b) trypsin-resistant core protein for parasitic Hymenoptera (*Brachymeria intermedia*), a beneficial parasite of the housefly (*Musca domestica).* Parasitic Hymenoptera exposed to trypsin-resistant CryIA(b) protein at a concentration of 20 ppm in honey/water solution for thirty days exhibited no treatment related mortality or signs of toxicity. The LC_{50} for CryIA(b) protein in parasitic Hymenoptera was greater than 20 ppm, the highest dose tested. The no-observal effect level was 20 ppm (Hoxter and Lynn, 1992b).

(d) Ladybird beetles

A study was performed to assess the safety of CryIA(b) trypsin-resistant core protein for ladybird beetles (*Hippodamia convergens*), a beneficial predaceous insect which feeds on aphids and other plant insects commonly found on stems and foliage of weeds and cultivated plants. Ladybird beetles exposed to trypsin-resistant CryIA(b) protein at a test concentration of 20 ppm in a honey/water solution for nine days exhibited no treatment related mortality or signs of toxicity. The LC_{50} for CryIA(b) protein in ladybird beetles was greater than 20 ppm, the highest dose tested. The no-observal effect level was 20 ppm (Hoxter and Lynn, 1992c).

(e) Resistance management

Since decades of experience have taught entomologists that insect populations could adapt to even the best insecticides, if those insecticides are not managed correctly, Monsanto has taken into consideration the risk of resistance of European corn borer populations to the CryIA(b) protein in Insect-Protected maize. Monsanto proposes and plans to implement a number of specific measures to minimize the risk of insect resistance development. These measures include:

A - Expanding the knowledge of insect biology and ecology.

B - Gene deployment strategy; e.g.: full-season, constitutive, optimal dose *CryIA(b)* expression to control insects heterozygous for resistance alleles. Conceptually, if a resistance occurs, it could be made functionally recessive by having a high CryIA(b) expression level that controls heterozygous insects for resistance alleles.

C - Refuges to support populations of CryIA(b) susceptible insects. Based on the concept that heterozygous insects for resistance alleles are controlled by high expression of the CryIA(b) protein, suceptible insects reservoirs will bring heterozygoty by mating with resistant homozygous insects.

D - Monitoring and reporting of any incidents of pesticide resistance development, to implement a contingeny plan in the area where resistance is detected.

E - Employment of integrated pest management practices that encourage ecosystem diversity and provide multiple tactics for insect control.

F - Communication and education plan, in order to get breeders, growers and distributors actively participating in the implementation of these strategies to minimize the risk of resistance development.

G - Development and deployment of products with alternative modes of action. This is related to genes encoding another insecticidal protein, with a different mechanism of action, in order to be combined with the *cryIA(b)* gene.

Monsanto is committed to developing and implementing these insect-resistance management strategies to preserve the efficacy of the CryIA(b) protein and maintain the sustainability of the Insect-Protected maize.

Not withstanding the implementation of these measures, the development of a resistance, if it occurs, will have no direct negative agri-environmental impact since the users would have to return to the current methods of control of ECB in maize.

The resistance management programme that Monsanto is implementing to minimize the risk of the development of ECB resistance to the CryIA(b) protein is discussed in further details in Appendix III.

2. Human health effects of the product

(a) Assessment of the safety of the CryIA(b) protein

The human safety assessment of CryIA(b) protein was organised into three groups of experiments. These included: 1) protein characterisation, 2) digestive fate studies in simulated gastric and intestinal fluids, and 3) acute oral toxicity in mice. Relatively large amounts of protein were required to conduct these experiments. Ideally, the test protein would be obtained from Insect-Protected maize. This was not feasible due to the low levels of expression and difficulties in purifying adequate quantities of protein from plant tissue. These studies were conducted with the trypsin-resistant core of the CryIA(b) protein since this is the insecticidally active portion of the CryIA(b) protein and the insecticidally active form of the CryIA(b) protein detected in Insect-Protected maize line MON 810 (Lee *et al.*, 1995b). The chemical and functional equivalence of the trypsin-resistant core of the CryIA(b) protein produced in *E. coli* and in Insect-Protected maize plants, including line MON 810, was established using a rigorous set of criteria including molecular weight, immunoreactivity and insecticidal activity

(Lee *et al.*, 1995b). The following sections summarise the protein characterisation and results of the digestive fate and acute oral toxicity studies for CryIA(b) protein.

(b) Toxicity assessment of CryIA(b) protein

CryIA(b) protein specificity. The CryIA(b) protein must be ingested by the insect to have an insecticidal effect (Huber and Lüthy, 1981*). The protein in its crystalline form is insoluble in aqueous solution at neutral or acidic pH (Bulla *et al.*, 1977*); however, the pH of the larval insect gut is alkaline which favours solubilisation of the protein crystal. The solubilised protein is subsequently activated by proteases in the insect gut. The insecticidal protein, which consists of approximately 600 amino acids, diffuses through the peritrophic membrane of the insect to the midgut epithelium, binding to the specific high affinity receptors on the surface of the midgut epithelium of target insects (Wolfersberger *et al.*, 1986*; Hofmann *et al.*, 1988a*). The gut becomes paralysed as a consequence of changes in electrolytes and pH in the gut causing the larval insect to quit feeding and die.

The CryIA(b) protein is insecticidal to only lepidopteran insects. Only seven of the eighteen insects screened were sensitive to *B. thuringiensis* subsp. *kurstaki* proteins (Macintosh *et al.*, 1990*) and all seven insects were lepidopterans. This specificity is directly attributable to the presence of receptors in the target insects (Van Rie *et al.*, 1990*; Hofmann *et al.*, 1988a*).

There are no receptors for the protein delta-endotoxins of *Bacillus thuringiensis* subspecies on the surface of mammalian intestinal cells, therefore, humans are not susceptible to these proteins (Hofmann *et al.*, 1988b*; Noteborn and Kuiper, 1995*; Sacchi *et al.*, 1986*). In addition to the lack of receptors for the *B.t.k.* proteins, the absence of adverse effects in humans is further supported by numerous reviews on the safety of the *B.t.* proteins (Ignoffo, 1973*; Shadduck, 1983*; Siegel and Shadduck, 1989*). We have conducted safety studies, described below, which show that the CryIA(b) protein shares no significant amino acid homology to know protein toxins, except for other *B.t.* proteins, that the CryIA(b) protein is rapidly degraded under conditions that simulate mammalian digestion and the CryIA(b) protein is not acutely toxic to mice.

Lack of homology of CryIA(b) protein to known protein toxins. The CryIA(b) protein does not show meaningful amino acid sequence homology when compared to known protein toxins present in the PIR, EMBL, SwissProt and GenBank protein databases, with the exception of other *B.t.* proteins (Astwood, 1995a*). The analysis of the homology of CryIA(b) protein to known protein toxins was based on the fact that patterns of amino acid sequence or regions of strong homology shared between two or more proteins may provide insight to the biological activity of the protein. Results from this search establish that, using the best methods available today, there are no biologically significant homologies between the full length CryIA(b) protein sequence and the protein sequence of all known toxins in the current protein databases.

Digestion of CryIA(b) protein in simulated gastric and intestinal fluids. The *E. coli* produced, trypsin-resistant core of the CryIA(b) protein was shown to be rapidly degraded *in vitro* using simulated digestive fluids (Ream, 1994). The trypsin-resistant core of the CryIA(b) protein was used in the simulated digestive study and for the acute gavage study since this is the insecticidally active form of the CryIA(b) protein (Huber and Lüthy, 1981*). In gastric fluid, the CryIA(b) protein degraded rapidly; more than 90% of the initially added CryIA(b) protein degraded within two minutes incubation in simulated gastric fluids as detected by western blot analysis. CryIA(b) protein bioactivity, as detected by insect bioassay, also dissipated readily; 74 to 90% of the added CryIA(b) activity dissipated within two minutes incubation in simulated gastric fluids, the earliest time point measured. To put the rapid degradation of the CryIA(b) protein in the simulated gastric system into perspective, approximately 50% of solid food has been estimated to empty from the human stomach within two hours, while liquid empties in approximately 25 minutes (Sleisenger and Fordtran, 1989*). In intestinal fluid, as expected, the CryIA(b) protein did not degrade substantially after 19.5 hours incubation as assessed by both western blot analysis and by insect bioassay. The tryptic core of this and other *Bacillus* insecticidal proteins have been shown to be relatively resistant to digestion by serine proteases like trypsin, a key protease in intestinal fluid (Bietlot *et al.*, 1989).

Acute mouse gavage study with CryIA(b) protein. An acute mouse gavage study the *E. coli* produced, trypsin-resistant core of the CryIA(b) protein was performed to directly assess potential toxicity associated with the CryIA(b) protein ([redacted]). Results from this study (Table C.1.) demonstrated, as expected, that the CryIA(b) protein is not toxic to mammals. Since the full length CryIA(b) protein expressed in maize is converted to the trypsin-resistant core upon digestion, the safety of the CryIA(b) protein was addressed by assessing the safety of the trypsin-resistant core. The *cryIA(b)* gene encoding the nature identical, full length CryIA(b) protein was introduced into *E. coli*. The full length protein produced was converted to the trypsin-resistant core, purified, characterised, and used in the acute mouse gavage study ([redacted]). CryIA(b) protein was administered by gavage to three groups of ten male and female mice. The targeted doses of CryIA(b) protein administered to mice were 0, 400, 1000, and 4000 mg/kg. The highest dose embodied the maximum hazard dose concept. Another group of mice was gavaged with a targeted dose of 4000 mg/kg bovine serum albumin (BSA). Mice were observed twice daily for signs of toxicity and food consumption was recorded daily. Food and water were provided *ad libitum*. All animals were sacrificed on post-dosing day 8 and 9 and subjected to gross necropsy. Approximately 40 tissues were collected and saved for each animal in the test. There were no statistically significant differences in mortality, body weights, cumulative body weight or total food consumption between the vehicle or BSA control groups and CryIA(b) protein-treated groups. A few incidental pathological changes were observed at necropsy which were randomly distributed among all groups and are commonly observed for the strain of mice used in laboratory testing. None of these findings were considered related to treatment. The only statistically significant difference observed was among the variances for

the groups in total food consumption. This difference was caused by the weight gained by a single mouse in the BSA control. Elimination of this one outlying data point resulted in no statistically significant difference in any of the groups for any of the parameters analysed.

Table C.1. Summary of the Results of the Acute Gavage Study Performed with the CryIA(b) Protein

Group	Target Dose (mg/kg)		Body Weight (g) Pretest	Day 7	Cumulative	Total Food Consumption (g/day)
Males						
Vehicle control	-	Mean	31 1	30 0	-0 34	5 3BT
Na Carbonate		Std. Dev	1.40	1 72	0 484	0 68
		Sample size	10	10	10	10
Vehicle control	4000	Mean	31.1	31 0	-0 18	6 2
BSA		Std. Dev	1.62	0.97	0 646	1 60
		Sample size	10	10	10	10
CryIA(b)	400	Mean	31.1	30.5	-0 58	5 3
		Std. Dev	1.39	1.18	0 702	1 23
		Sample size	10	10	10	10
CryIA(b)	1000	Mean	31.0	31.1	0 09	5 3
		Std. Dev.	1.55	1 64	1 111	0 40
		Sample size	10	10	10	10
CryIA(b)	4000	Mean	31.0	30.5	-0 53	5 5
		Std. Dev	1.47	1 87	1.006	1 12
		Sample size	10	10	10	10
Females						
Vehicle control	-	Mean	25 5	25.1	-0 38	6 4
Na Carbonate		Std. Dev	1.50	1.53	1 039	2 14
		Sample size	10	10	10	10
Vehicle control	4000	Mean	25.4	24 7	-0 64	7 3
BSA		Std. Dev	1.46	1 39	0 691	2 34
		Sample size	10	9	9	9
CryIA(b)	400	Mean	25.4	25 2	-0 16	8 0
		Std. Dev	1.47	2 13	1 198	3 69
		Sample size	10	10	10	10
CryIA(b)	1000	Mean	25.3	25.0	-0 38	8 0
		Std. Dev.	1.64	1.36	0 965	2 32
		Sample size	10	10	10	10
CryIA(b)	4000	Mean	25 5	25.5	0.04	6 8
		Std Dev	1.65	1 70	1 276	2 65
		Sample size	10	10	10	10

* -- Dunnett's test (two-tailed) indicates statistically significant difference (P≤ 0 05)
** -- Dunnett's test (two-tailed) indicates statistically significant difference (P≤ 0 01)
BT -- Bartlett's test indicates statistically significant difference among variances of the different groups (P≤ 0 01)
NA -- Dunnett's test not appropriate for this group/sex/date

In summary, CryIA(b) protein shows no amino acid sequence similarity to known protein toxins, other than other *B.t.* proteins, and the CryIA(b) protein is rapidly degraded and its insecticidal activity lost under conditions that simulate mammalian digestion. Furthermore, there were no indications of toxicity as measured by treatment related adverse effects in mice administered CryIA(b) protein by oral gavage. These studies support the safety of CryIA(b) protein and are fully consistent with the history of safe use for the CryIA(b) protein which has been demonstrated as highly selective for insects, with no activity against other types of living organisms such as mammals, fish, birds, or invertebrates (EPA, 1988*).

(c) Assessment of the allergenic potential of the CryIA(b) protein

Large quantities of a vast variety of proteins are consumed orally by humans each day. Rarely do any of these tens of thousands of proteins elicit an allergenic response (Taylor, 1992*). The most important factor to consider in assessing allergenic potential is whether the source of the gene being introduced in to plants is allergenic (FDA, 1992*). *Bacillus thuringiensis* [the source of the *cryIA(b)* gene] has no history of causing allergy. In over 30 years of commercial use, there have been no reports of allergenicity to *Bacillus thuringiensis,* including occupational allergy associated with manufacture of products containing *Bacillus thuringiensis* (EPA, 1995). In addition, the biochemical profile of the CryIA(b) protein provides a basis for allergenic assessment when compared with known protein allergens. Protein allergens must be stable to the peptic and tryptic digestion and the acid conditions of the digestive system if they are to reach and pass through the intestinal mucosa to elicit an allergenic response. Another significant factor contributing to the allergenicity of proteins is their high concentration in foods that elicit an allergenic response (Taylor, 1992*; Taylor *et al.* 1987*; and Taylor *et al.*, 1992*).

A comparison of the amino acid sequence of an introduced protein with the amino acid sequences of known allergens is a useful indicator of allergenic potential. The amino acid sequences of most major allergens, including food allergens, have been reported (King *et al.*, 1994*), but the list is likely to expand with time. The important IgE binding epitopes of many allergenic proteins have been mapped (Elsayed and Apold, 1983*; Elsayed *et al.*, 1991*; Zhang *et al.*, 1992*). The optimal peptide length for binding is between 8 and 12 amino acids (Rothbard and Gefter, 1991*). T-cell epitopes of allergenic proteins and peptide fragments appear to be least 8 amino acids in length (O'Hehir *et al.*, 1991*). Exact conservation of epitope sequences is observed in homologous allergens of disparate species (Astwood *et al.*, 1995*). Indeed, conservative substitutions introduced by site-directed mutagenesis reduce epitope efficacy (Smith and Chapman, 1995*). Based on this information, an immunologically relevant sequence comparison test for similarity between the amino acid sequence of the introduced protein and known allergens is defined as a match of at least eight contiguous identical amino acids is required.

The amino acid sequences of the 219 allergens present in public domain genetic databases (GenBank, EMBL, PIR, and SwissProt) have been searched for similarity to the amino acid sequence of CryIA(b) protein using the FASTA computer program (Pearson and Lipman, 1988*). No biologically significant homology (Doolittle, 1990*) and no immunologically significant sequence similarities were observed with these allergens (Astwood, 1995b*). We conclude (1) that the cryIA(b) gene introduced into maize does not encode known allergens, and (2) that the introduced protein does not share immunologically significant amino acid sequences with known allergens.

CryIA(b) protein does not possess any of the other characteristics common to protein allergens. The CryIA(b) protein was shown to be very labile to digestion by the proteases present in the mammalian digestive system (Section C.2. above), minimizing any potential for these proteins to be absorbed by the intestinal mucosa, if consumed. *In vitro,* simulated mammalian gastric and intestinal digestive mixtures were established and used to assess the susceptibility of the CryIA(b) protein to proteolytic digestion. The method of preparation of the simulated digestion solutions used is described in the United States Pharmacopeia (1989), a frequently cited reference for *in vitro* digestion studies. The ability of food allergens to reach and to cross the mucosal membrane of the intestine are likely prerequisites for allergenicity. Clearly, a protein which is stable to the proteolytic and acidic conditions of the digestive tract has an increased probability of reaching the intestinal mucosa. Many allergens exhibit proteolytic stability (King *et al.*, 1967*; Kortekangas-Savolainen *et al.*, 1993*; Onaderra *et al.*, 1994*; Taylor, 1992*; Taylor *et al.*, 1987*; Metcalfe, 1985*), although the majority remain untested directly. Intact proteins are capable of crossing the mucosal membrane of the gut and of entering the circulatory system (Gardner, 1988*). Thus, physicochemical properties which favor digestive stability can be used as an important indicator of allergenic potential.

The data from the simulated digestion experiments demonstrated that the CryIA(b) protein degraded rapidly; more than 90% of the initially added CryIA(b) protein degraded after two minutes incubation in the gastric system (Ream, 1994). As expected, in the intestinal system, the full length CryIA(b) protein was rapidly converted to the trypsin-resistant core, which was not further degraded. To put the rapid degradation of this protein in the simulated gastric system into perspective, solid food has been estimated to empty from the human stomach by about 50% in two hours, while liquid empties 50% in approximately 25 minutes (Sleisenger and Fordtran, 1989*). Therefore, any CryIA(b) protein consumed would be rapidly degraded in the gastric system.

Finally, most allergens are present as major protein components in the specific food. This is true for the allergens in milk (Baldo, 1984*; Lebenthal, 1975*; Taylor, 1986*; Taylor *et al.*, 1987*), soybean (Shibasaki *et al.*, 1980*; Burks *et al.*, 1988*; Pedersen and Djurtoft, 1989*), and peanuts (Barnett *et al.*, 1983*; Sachs *et al.*, 1981*; Barnett and Howden, 1986*; Kemp, 1985*). In contrast to this generality for common allergenic proteins, the CryIA(b) protein is present in the

grain of Insect-Protected maize lines MON 810 at low levels. The CryIA(b) protein is present in line MON 810 at approximately 0 3 to 0.5 μg/g fresh weight of the maize seed (Table B.7., B.8., B.9.). The low level of the CryIA(b) protein in maize seed, combined with the digestive lability of this protein relative to that for known food allergens establishes an extremely low probability of the CryIA(b) protein being absorbed via the intestinal mucosa during consumption and triggering production of antibodies including the IgE antibodies responsible for allergenicity.

The trypsin resistant insecticidal core of CryIA(b) protein expressed in these Insect-Protected maize plants is identical to the portion of the CryIA(b) protein contained in microbial formulations that have been used safely commercially for over 30 years (Lee *et al.*, 1995a). These microbial formulations have been used on a wide variety of crops, including fresh vegetables, with no reported allergenic responses, establishing a sound basis for the lack of allergenic concern for the CryIA(b) protein.

In summary, the data and analyses described above and summarized in Table C.2. support the conclusion that the CryIA(b) protein is not derived from allergenic sources, does not possess immunologically relevant sequence similarity with known allergens, and does not possess the characteristics of known protein allergens. Furthermore, this protein or closely related proteins have a history of use with no allergenic concerns. This information, coupled with the extremely rapid digestion of this protein under *in vitro* digestive conditions that mimic human digestion, established that, using the best methodology available today, there is no reason to believe that this protein should pose any significant allergenic risks for consumption of the products produced from Insect-Protected maize.

Table C.2. Characteristics of Known Allergenic Proteins[a]

Characteristic	Allergens	CryIA(b)
Allergenic source of gene	yes	no
Similar sequence to allergens	yes	no
Stable to digestion	yes	no
Prevalent protein in food	yes	no

[a] As described in Taylor (1992) and Taylor *et al.* (1987)

(d) Compositional analyses of Insect-Protected maize

Extensive compositional analyses were performed on grain from Insect-Protected maize line MON 810 obtained from the 1994 U.S field trials (Tables C.3 through C.13). Compositional analyses were also performed on grain and forage collected from the 1995 European field trials (Tables C.7 through C.9). In addition, compositional analyses were performed on grain and forage collected from progeny of MON 810, hybrid varieties that are representative of the hybrids to be introduced commercially (Tables C 10 through C.13).

The compositional analysis of grain was performed by AOAC methods (1990), while forage was analyzed by both AOAC and NIR (Near-Infrared Reflectance Spectroscopy) methodologies. All of the analyses conducted by AOAC methods were performed at Corning Hazleton, Inc. on freshly ground grain and forage samples. Processed, dried forage samples were analyzed by NIR at Pioneer Hi-Bred Intl. in Johnston, Iowa for neutral detergent fiber, acid detergent fiber, crude fiber, crude protein, ash, *in vitro* digestibility (cellulase method) (IVDC), soluble sugars, dry matter, and *in situ* dry matter disappearance (ISDMD).

i. Compositional analyses of grain from line MON 810 plants Grown in 1994 US field trials

The major components of the grain for maize line MON 810 and control, MON 818 were analysed under Good Laboratory Practices (GLP) on grain harvested from 6 U.S. GLP field trials in 1994 (Sanders *et al.*, 1995). The control line, MON 818, is similar in pedigree to line MON 810 [(((Hi-IIxB73) selfed)xMo17) selfed] but is not an isogenic control because of the variability in the parental High-Type II line. Proximates (protein, fat, ash, carbohydrates, calories and moisture), amino acid composition and fatty acid profile were performed on the ground grain by published methods (Sanders and Patzer, 1995). The values reported for the compositional analyses at Corning Hazleton Inc. (Madison, Wisconsin, USA) were expressed as percent dry weight of the sample, correcting for the measured moisture content.

A. Proximate analysis of maize grain

The levels of the major components of maize grain (protein, fat, ash, carbohydrates, calories and moisture) are summarised in Table C.3. The levels of protein, fat, ash, carbohydrates, calories and moisture were similar for line MON 810 and the control line, MON 818. The values for both lines were also within the published and reported literature ranges for all components measured.

Table C.3. Summary of Proximate Analysis of Grain from Maize Line MON 810

Characteristic	MON 818 Control		MON 810 Test		Literature	
	Mean[b]	Range[c]	Mean[b]	Range[c]	Range	Range[d]
Protein	12.8	11 7-13 6	13.1	12 7-13 6	6.0-12 0[e] 9 7-16.1[f]	11.2-12.9
Fat[a]	2 9	2 6-3 2	3 0	2 6-3 3	3 1-5 7[e]	3 8-4.2
Ash[a]	1 5	1 5-1 6	1 6	1 5-1 7	1 1-3 9[e]	1.5-1.8
Carbohydrate[a]	82.7	81 7-83 8	82.4	81 8-82 9	not reported	81.7-83 0
Calories/100g[a]	409	406-410	408	407-410	not reported	412-416
Moisture %	12.0	10 6-14.2	12.4	11 0-14.4	7-23[e]	13 0-15.8

[a]. Percent dry weight of sample.
[b] Value reported is mean of six samples, one from each field site.
[c]: The range denotes the lowest and highest individual values across sites for each line
[d]: Sanders and Patzer (1995), range for a control with similar genetic background.
[e]: Watson, 1987.
[f]: Jugenheimer, 1976

B. Amino acid composition of MON 810 maize grain

The results of the analysis of the amino acid composition on maize grain samples for both lines MON 810 and MON 818 are presented in Table C.4. The values for each amino acid (mg/g) were converted to percent of total protein. The values for all amino acids were typical of the values reported in the literature (Watson, 1982*) and for a control maize line with a similar genetic background (Sanders and Patzer, 1995).

Table C.4. Amino Acid Composition of Maize Grain[a]

Amino Acid	MON 818 Control % of Total Protein Mean[d]	MON 818 Control % of Total Protein Range[e]	MON 810 Test % of Total Protein Mean[d]	MON 810 Test % of Total Protein Range[e]	Literature Range[b] %	Reported Range[c] %
Nutritionally essential						
Methionine	1.7	1.6-1.7	1.7	1.6-1.9	1.0-2.1	2.0-2.6
Cystine	1.9	1.8-2.0	2.0	1.9-2.1	1.2-1.6	1.9-2.3
Lysine	2.8	2.7-2.9	2.8	2.5-2.9	2.0-3.8	2.9-3.4
Tryptophan	0.6	0.4-0.6	0.6	0.5-0.7	0.5-1.2	0.5-0.6
Threonine	3.8	3.7-3.9	3.9	3.7-4.4	2.9-3.9	4.0-4.2
Isoleucine	3.8	3.6-4.0	3.7	3.3-4.1	2.6-4.0	3.7-3.8
Histidine	2.9	2.8-3.0	3.1	2.9-3.3	2.0-2.8	3.0-3.3
Valine	4.6	4.3-4.8	4.5	4.1-4.9	2.1-5.2	4.5-4.8
Leucine	14.5	13.8-15.0	15.0	14.1-16.7	7.8-15.2	13.6-13.8
Arginine	4.5	4.2-4.7	4.5	4.1-4.7	2.9-5.9	4.4-5.0
Phenylalanine	5.4	5.2-5.6	5.6	5.4-6.1	2.9-5.7	5.2-5.4
Glycine	3.7	3.5-3.8	3.7	3.4-4.0	2.6-4.7	3.9-4.2
Non-essential						
Alanine	7.8	7.5-8.0	8.2	7.8-8.9	6.4-9.9	7.8-8.1
Aspartic acid	6.6	6.3-6.8	7.1	6.4-8.2	5.8-7.2	6.8-7.3
Glutamic acid	21.1	20.1-21.6	21.9	20.4-24.4	12.4-19.6	19.9-20.9
Proline	9.6	9.4-9.8	9.9	9.7-10.5	6.6-10.3	9.0-9.4
Serine	5.2	5.1-5.4	5.5	5.3-5.9	4.2-5.5	5.5-6.0
Tyrosine	4.0	3.9-4.1	4.4	4.1-4.8	2.9-4.7	3.8-4.3

[a] Values are expressed as percent of total protein.
[b] Watson, 1982. Values are per cent of total protein [10.1% total protein (Nx6.25)]
[c] Sanders and Patzer (1995), range for a control with similar genetic background
[d] Value reported is mean of six samples, one from each field site (Sanders *et al.*, 1995).
[e] Range denotes the lowest and highest individual values across sites for each line

C. Fatty acid composition of MON 810 maize grain

The values for fatty acid composition of maize grain from line MON 810 and the control line, MON 818 are summarised in Table C.5. Results are reported for the fatty acids which gave detectable values in the assay. The fatty acid values were similar between line MON 810 and control line, MON 818 and typical of the values previously reported in the literature (Watson, 1982*) and for a control maize line with a similar genetic background (Sanders and Patzer, 1995). The fatty acids which were not detectable in the assay were: caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, heptadecanoic, eicosadienoic, eicosatrienoic and arachidonic.

Table C.5. Fatty Acid Composition of Maize Grain[a]

Component	MON 818 Control Mean[b]	MON 818 Control Range[c]	MON 810 Test Mean[b]	MON 810 Test Range[c]	Literature[d] Range	Reported Range[e]
Linoleic (18:2)	63.0	61.8-64.6	62.6	59.5-64.7	35-70	61.7-65.0
Oleic (18:1)	22.8	21.6-23.9	23.2	21.5-25.4	20-46	21.3-23.6
Palmitic (16:0)	10.5	10.2-10.7	10.5	10.2-11.1	7-19	10.2-10.8
Stearic (18:0)	1.8	1.8-1.9	1.9	1.7-2.1	1-3	1.6-2.1
Linolenic (18:3)	0.9	0.8-0.9	0.8	0.7-0.9	0.8-2	0.9-1.1

[a]. Value of fatty acid is % of total lipid. Other fatty acids were below the limit of detection of the assay
[b]: Values presented are means (six samples for each line).
[c]: Range denotes the lowest and highest individual value across sites for each line
[d]: Watson, 1982.
[e]: Sanders and Patzer (1995), range for a control with similar genetic background

In summary, compositional data for protein, fat, ash, carbohydrates, calories, moisture, amino acids and fatty acids for line MON 810 was comparable to the control line, MON 818, and within the published and reported literature ranges for commercial hybrids. Based on these data, it was concluded that the grain from the maize line MON 810 and the control line, MON 818 are similar in composition and representative of maize grain currently in commerce.

ii. Compositional analyses of grain from line MON 810 from plants grown in the 1995 European field trials

The major components of the grain for maize line MON 810 and control, MON 820 were analysed under Good Laboratory Practices (GLP) on grain harvested from three field trials conducted in 1995 in France. The control line, MON 820, is similar in pedigree to line MON 810 but is not an isogenic control because of the variability if the parental High-Type II line. Proximates (protein, fat, ash, carbohydrates, calories and moisture), amino acid composition and fatty acid profile were performed on the ground grain by published methods (Sanders and Patzer, 1995). The values reported for the compositional analyses performed at Corning Hazleton Inc. (Madison, Wisconsin, USA) were expressed as percent dry weight of the sample, correcting for the measured moisture content.

A. Proximate analysis of maize grain

The levels of the major components of maize grain (protein, fat, ash, carbohydrates, calories and moisture) are summarised in Table C.6. The levels of protein, fat, ash, carbohydrates, calories and moisture were similar for line MON 810 and the control line, MON 820. The values for both lines were also within the published and reported literature ranges.

Table C.6. Summary of Proximate Analysis of Grain from Maize Line MON 810 Harvested From France Field Trials

	MON 820 Control		MON 810 Test		Literature	
Characteristic	**Mean[b]**	**Range[c]**	**Mean[b]**	**Range[c]**	**Range**	**Range[d]**
Protein	10 7	9 03-11.7	11 5	10 5-12 2	6 0-12.0[e]	11.2-12.9 9 7-16.1[f]
Fat[a]	3 0	2.4-3.3	3.0	2 8-3 3	3 1-5 7[e]	3 8-4.2
Ash[a]	1.4	1.3-1.6	1 4	1 3-1 5	1 1-3 9[e]	1 5-1 8
Neutral detergent fiber	12.3	9.6-15 3	12.1	10 7-13 9	8.3-11 9[e]	not reported
Acid detergent fiber	3 6	3 1-3 9	3 4	2 7-4 1	3 3-4 3[e]	not reported
Carbohydrates[a]	84 9	83.7-86.1	84.1	83 1-84 8	not reported	81 7-83 0
Moisture %	12 1	11 7-12.3	13 3	12 1-15 2	7-23[e]	13.0-15 8

[a] Percent dry weight of sample
[b] Value reported is mean of three samples, one from each field site
[c] Range denotes the lowest and highest individual values across sites for each line
[d] Sanders and Patzer (1995), range for a control with similar genetic background.
[e] Watson, 1987.
[f] Jugenheimer, 1976.

B. Amino acid composition of MON 810 maize grain

The results of the analysis of the amino acid composition on maize grain samples for both lines MON 810 and MON 820 from the France field trials are presented in Table C.7. The values for each amino acid (mg/g) were converted to percent of total protein. The values for all amino acids were typical of the values previously reported in the literature (Watson, 1982*) and for a control maize line with a similar genetic background (Sanders and Patzer, 1995).

Table C.7. Amino Acid Composition of Maize Line MON 810 Grain Harvested from France Field Trials[a]

	MON 820		MON 810		Literature Range[b]	Reported Range[c]
	% of Total Protein		% of Total Protein		%	%
Amino Acid	Mean[d]	Range[e]	Mean[d]	Range[e]		
Nutritionally essential						
Methionine	1.5	1.4-1 7	1 4	1 4-1.5	1.0-2.1	2.0-2 6
Cystine	2.1	1.9-2.4	1 9	1 9-2 1	1 2-1 6	1 9-2 3
Lysine	3.2	3.1-3 5	2 9	2 7-3 1	2.0-3 8	2 9-3.4
Tryptophan	0.6	0.5-0 6	0 5	0 4-0 5	0 5-1.2	0 5-0.6
Threonine	3 7	3 7-3 8	3 7	3 6-3 7	2 9-3 9	4.0-4.2
Isoleucine	3 9	3 7-4.3	3 8	3 4-4 3	2 6-4 0	3 7-3.8
Histidine	3.0	2 9-3 1	3 0	2 9-3 0	2 0-2 8	3 0-3 3
Valine	4.8	4 7-4.9	4 7	4 4-4 9	2.1-5 2	4.5-4.8
Leucine	14.2	13.5-15 0	14 5	13 9-15 3	7 8-15 2	13 6-13 8
Arginine	4.1	3 9-4 3	3 9	3 6-4 1	2.9-5 9	4 4-5 0
Phenylalanine	5.6	5.3-5 9	5 6	5 4-5 9	2 9-5.7	5 2-5.4
Glycine	3.7	3 5-3.9	3 5	3 4-3 7	2 6-4 7	3 9-4 2
Non-essential						
Alanine	8.1	7 8-8.5	8.2	7 9-8 4	6.4-9.9	7 8-8.1
Aspartic acid	7.0	6 6-7 3	7 1	6.9-7 3	5 8-7.2	6.8-7 3
Glutamic acid	21.0	20 1-21 8	21 3	20 8-21.8	12.4-19 6	19.9-20.9
Proline	9 6	9 2-10 0	9 7	9 5-9 9	6 6-10 3	9.0-9.4
Serine	5.4	5.3-5 5	5 5	5 4-5 6	4 2-5 5	5.5-6 0
Tyrosine	4.0	3 7-4.3	4 0	3 9-4 2	2 9-4 7	3.8-4 3

[a] Values are expressed as percent of total protein.
[b] Watson, 1982 Values are per cent of total protein [10.1% total protein (Nx6 25)]
[c] Sanders and Patzer (1995), range for a control with similar genetic background.
[d] Value reported is mean of three samples, one from each field site.
[e] Range denotes the lowest and highest individual values across sites for each line.

C. Fatty acid composition of MON 810 maize grain

The values for fatty acid composition of maize grain from line MON 810 and the control line, MON 820 from the France field trials are summarised in Table C.8. Results are reported for the fatty acids which gave detectable values in the assay. The fatty acid values were similar between line MON 810 and control line, MON 820. The values for both the lines were typical of the values previously reported in the literature (Watson, 1982*) and for a control maize line with a similar genetic background (Sanders and Patzer, 1995). The fatty acids which were not detectable in the assay were: caprylic, capric, lauric, myristic, myristoleic, pentadecanoic, heptadecanoic, eicosadienoic, eicosatrienoic and arachidonic.

Table C.8. Fatty Acid Composition of Maize Line MON 810 Grain Harvested from France Field Trials[a]

Component	MON 820		MON 810		Literature[d] Range	Reported
	Mean[b]	Range[c]	Mean[b]	Range[c]		Range[e]
Linoleic (18:2)	63 5	62 7-64.4	64.0	63 3-64.6	35-70	61 7-65.0
Oleic (18:1)	22.6	21.8-23.5	22.0	21.0-22 9	20-46	21 3-23 6
Palmitic (16:0)	10 4	10 2-10.7	10 5	10.3-10.8	7-19	10 2-10 8
Stearic (18:0)	1 6	1 5-1 6	1.5	1.4-1 7	1-3	1 6-2.1
Linolenic (18:3)	1 0	1.0-1.1	1.1	1 0-1 1	0.8-2	0 9-1.1

[a] : Value of fatty acid is % of total lipid. Other fatty acids were below the limit of detection of the assay.
[b] : Values presented are means (three samples for each line).
[c] : Range denotes the lowest and highest individual value across sites for each line
[d] : Watson, 1982
[e] : Sanders and Patzer (1995), range for a control with similar genetic background.

In summary, compositional data for protein, fat, ash, carbohydrates, moisture, amino acids and fatty acids for line MON 810 was comparable to the control line, and within the published and reported literature ranges for commercial hybrids. Based on these data, it was concluded that the grain from the maize line MON 810 and the control line are similar in composition and representative of maize grain currently in commerce.

iii. Compositional analyses of forage from plants from maize line MON 810 grown in the 1995 European field trials

The major components of the forage for maize line MON 810 and control, MON 820 were analysed under Good Laboratory Practices (GLP) on forage plants harvested from three field trials in France. Proximates (protein, fat, ash, carbohydrates, calories and moisture), acid detergent fiber (ADF) and neutral detergent fiber (NDF) were performed on the ground forage plants by published methods (AOAC, 1990; Williams and Norris, 1987). The values reported for the compositional analyses as measured by the AOAC method at Corning Hazleton Inc. (Madison, Wisconsin, USA) and by NIR method (Table C.9) were expressed as percent dry weight of the sample, correcting for the measured moisture content.

Table C.9. Compositional Analyses on Forage Samples of Maize Line MON 810 from France Field Trials[a]

	AOAC[b]		NIR Analysis[c]	
	MON 820	MON 810	MON 820	MON 810
Protein %	4 7-7 4	5.7-8.4	4 8-8 2	6.2-8 5
Ash %	2 9-4.4	3.1-3.6	3 9-4 6	3 9-4.6
ADF %	25.6-29 2	22.6-27.2	27 4-29.8	24 6-26.6
NDF %	39 9-43.3	36.9-41 4	53 5-55.9	49.3-51.3
Total fat %	1 4-2.1	1.3-1 7	not done	not done
Carbohydrates, %	88 0-89.1	86.9-89.8	not done	not done
Dry Matter %	26.5-31.3	28.7-32.4	91 4-94.1	92 8-95.1

[a] : There were three samples, one from each field site. Values are ranges, the lowest and highest individual value across sites for each line.

[b] : Association of Official Analytical Chemists, 1990.

[c] : Near-Infrared Reflectance Spectroscopy, Williams and Norris, 1987 Samples were dried prior to analysis.

In summary, compositional data for protein, fat, ash, acid detergent fiber, neutral detergent fiber, fat, carbohydrates and dry matter for maize line MON 810 was similar to the control line, MON 820. Based on these data, it was concluded that the forage from the maize line MON 810 and the control line, MON 820 are similar in composition.

iv. Compositional analyses of progeny of MON 810

Field trials were conducted in both Italy and France in 1995 to produce forage and grain samples for the compositional analysis of Insect-Protected maize hybrids of line MON 810. Five Insect-Protected maize hybrids were developed through crossing of the MON 810 event. Nonmodified versions of the same hybrids were used as controls. Field trials were approved under permit number B/IT/95-23 in Italy and permit number 95.03.06 in France.

Grain from four or five plants of each insect protected maize hybrid and control hybrid was pooled by site. ground to a fine powder and analyzed by Corning Hazleton, Inc. The samples were analyzed by AOAC methodology (Association of Official Analytical Chemists) for protein, ash, acid detergent fiber (ADF), neutral detergent fiber (NDF), fat, moisture content, amino acid composition and fatty acid profile according to published methods (AOAC, 1990).

Forage plant samples were collected from the field trials conducted in Italy and France. One or two plants of each Insect-Protected maize hybrid or control hybrid were pooled and processed by standard procedures. Processed, dried samples were analyzed by NIR for neutral detergent fiber, acid detergent fiber, crude fiber, crude protein, ash, in vitro digestibility (cellulase method), soluble sugars, dry matter, and in situ dry matter disappearance. All analyses were conducted at Pioneer Hi-Bred Intl. in Johnston, Iowa. Results are reported as percentages of the dry weight of the sample. The forage samples were analyzed on a NIR Systems 6500 scanning near infrared spectrometer. The spectra were recorded from 1100 to 2500 nanometers. All spectra were measured in the reflectance mode. Calibrations used for prediction of constituent values for both grain and forage samples were internal calibrations developed by Pioneer Hi-Bred Intl. In addition, the forage samples were analyzed by AOAC methods at Corning Hazleton, Inc.

The compositional analysis data presented in Tables C.10 through C.13 are expressed as ranges. The range represents the minimum and maximum levels measured across the Insect-Protected or control hybrids at a given location. Many of the nutritional characteristics measured in these studies are known to vary widely across hybrids and environments (Perry, 1988). Therefore, it is most informative to analyze a range of values for nutritional characteristics when reporting results of such studies.

A. Proximate analysis on grain from progeny of maize line MON 810

The results of proximate analyses as performed by AOAC methodology on grain of maize line MON 810 are summarised in Table C.10. The values for all parameters are similar for the control hybrid and MON 810 hybrids, within and between the field sites. The measured ranges are similar to the published literature ranges (Watson, 1987*; Table C.6).

Table C.10. Proximate Analyses on Grain from Progeny of Maize Line MON 810

	Italy site Ranges		France site Ranges	
	Control	MON 810	Control	MON 810
Protein %	9 1-10.4	8 4-11.0	10.1-11.2	10.7-13 7
Ash %	1.3-1.6	1.4-1.6	1 3-1 5	1.3-1.7
ADF %	2 1-4.1	2.2-3.2	2.3-2 9	3 1-3 6
NDF %	8 0-9.7	7.7-9.5	7.2-9.4	8.5-9.4
Total fat %	3 1-3 8	3.6-4 8	3 3-4 3	3.2-4 9
Carbohydrates, %	84.2-86.4	82.9-86.4	83 4-85.3	79.8-84 7
Calories C/100g	410-412	412-418	412-416	411-418
Dry Matter %	87.7-89.7	87.1-89.4	78 0-80 5	63.5-78 9

B. Amino acid composition of grain from progeny of maize line MON 810

The amino acid composition of grain from progeny of maize line MON 810 and the control is summarised in Table C.11. The range of values for each amino acid are similar for the control and MON 810 hybrids. The values are also similar to those reported in the literature (Watson, 1982*; Table C.7).

Table C.11. Amino Acid Composition of Grain from Progeny of Maize Line MON 810

Amino Acid	Italy site Range[a] % of Total Protein Control	Italy site Range[a] % of Total Protein MON 810	France site Range[a] % of Total Protein Control	France site Range[a] % of Total Protein MON 810
Nutritionally essential				
Methionine	1.8-2 1	1.7-2.2	1 8-2 0	1.7-2.3
Cystine	2.1-2.3	2.1-2 3	1 9-2.2	1.9-2 3
Lysine	3.1-3.4	2.9-3.6	2 9-3 3	2.8-3.2
Tryptophan	0 6-0.7	0.6-0 7	0 5-0.6	0.6-0 6
Threonine	3 8-3.9	3.5-3.9	3 7-3 9	3.6-4 0
Isoleucine	3 6-4.4	3.7-4.5	3 9-4.3	3 9-4 7
Histidine	2 7-3 1	2.9-3 1	2 7-3 0	2 8-3 1
Valine	4 4-5.0	4.6-5.0	4 3-5 1	4 8-5 3
Leucine	13 3-13.6	12.8-13.6	13 4-14.2	14.0-14.8
Arginine	4 4-4.8	4.3-5.0	3 9-4 3	3 9-4 4
Phenylalanine	5.3-5.8	5.3-5.8	5 5-5.7	5 6-6 0
Glycine	3.8-4.0	3.7-4.2	3 6-3 8	3 5-4 0
Non-essential				
Alanine	7.7-8.2	7.6-8.0	7 7-8 2	7 9-8 6
Aspartic acid	6.7-7.2	6.7-7.2	6 5-7.1	6 4-7 3
Glutamic acid	20.0-20.4	19.2-20.1	19 9-21.0	20 7-21 6
Proline	9 1-9.7	9.3-10.0	9 0-9 7	9 5-9 9
Serine	5 2-5 7	5.0-5.3	5 1-5.7	5 2-5 6
Tyrosine	4 2-4.3	4.1-4.5	4 2-4 6	4 3-4.8

[a] Range denotes the lowest and highest individual values across 4 hybrids at each site

C. Fatty acid profile of grain from progeny of maize line MON 810

The fatty acid profile of grain from progeny of line MON 810 is summarised in Table C.12. The range of values for each fatty acid are similar for the control hybrids and the MON 810 hybrids and were within the reported literature (Watson, 1982*, Table C.8).

Table C.12. Fatty Acid Profile of Grain from Progeny of Maize Line MON 810[a]

Component	Italy site Range[b]		France site Range[b]	
	Control	MON 810	Control	MON 810
Linoleic (18:2)	53.2-60.6	62.2-65.8	55.3-60 7	61.8-65.2
Oleic (18:1)	23 8-32.3	20 1-24.2	23.9-30 0	20 6-24.2
Palmitic (16:0)	10 6-12 2	10 1-11 6	10.7-12 3	10 4-11.8
Stearic (18:0)	1.3-1.5	1 4-1.6	1.4-1.6	1 5-1 6
Linolenic (18:3)	1.2-1 5	1 1-1.3	1 2-1 4	1 0-1 2
Arachidic (20:0)	0.3-0 4	0.3-0.4	0 3-0 4	0 3-0 4
Eicosenoic (20:1)	0 3-0 4	0 3-0.4	0 3-0 4	0 3-0 4
Behenic (22:0)	0 1-0.2	0.1-0.2	0.1-0.2	0 1-0.2

[a] : Value of fatty acid is % of total lipid. Other fatty acids were below the limit of detection of the assay. There were 5 control hybrids and 5 MON 810 hybrids

[b] : Range denotes the lowest and highest individual value across all hybrids.

D. Compositional analyses on forage from progeny of maize line MON 810

Tables C.13 (a and b) summarize the results of the compositional analysis of forage samples of the control and MON 810 hybrids. Table C.13a summarises the data from NIR analysis, Table C.13b contains the AOAC data. All data are expressed on a dry weight basis.

Table C.13a. Near-Infrared Reflectance (NIR) Spectroscopy Results on Forage from Progeny of Maize Line MON 810

	Italy Site Range[a]		France Site Range[a]	
	Control	MON 810	Control	MON 810
Crude Protein	7.4-8 6	8 0-9.4	7 4-8.4	7 3-8.3
Ash	4.8-5 7	4.5-5.4	3 5-4.6	3 8-5.3
Crude Fiber	21.4-25 1	19 0-22 6	19 3-24 6	20 4-23 7
ADF[b]	25.2-30 7	24.1-28.0	22 0-28.6	23.3-27 7
NDF[c]	47.8-54 4	46 3-51.2	43.2-54.0	45 3-51 8
Starch	15.4-28 6	22 2-30 6	12.9-32.2	8.2-24.4
Soluble Sugars	7 4-18.6	6.5-15.7	14 9-23 7	21 0-28.1
Dry Matter	94.8-95 1	92.6-95 5	93 2-95 1	94 2-95 2
IVDC[d]	65.6-71 5	69 2-73.0	69.1-76 5	70 9-76 1
ISDMD[e]	36.8-40 9	38.1-41.9	38 7-42 9	40 2-44 3

[a] : Range denotes the lowest and highest individual values of 5 hybrids tested.
[b] : Acid detergent fiber.
[c] : Neutral detergent fiber
[d] : *in vitro* digestibility-cellulose method.
[e] : *in situ* dry matter disappearance.

Table C13b. AOAC Results on Forage from Progeny of Maize Line MON 810

	Italy site Range[a]		France site Range[a]	
	Control	MON 810	Control	MON 810
Protein %	6 3-7 3	6 5-7 7	6 4-8 8	5 9-7 6
Ash %	3.8-4.8	4.0-4.7	2 7-3 4	2 7-3 9
ADF[b] %	19.7-29 0	17.3-22.4	19 6-25 2	18 0-20 5
NDF[c] %	31 5-35.5	29.7-33 7	30 0-35 3	29 5-32 8
Total fat %	1.7-2.4	1.8-2.5	1 2-2 1	1 0-2 1
Carbohydrates, %	86.1-87.8	85.4-87 1	88 2-89.0	87 1-89 8
Calories C/100g	390-394	390-395	395-398	393-398
Dry Matter %	29.0-34.5	29.6-36.2	33 7-38.3	34.2-40.0

[a] : Range denotes the lowest and highest individual values of 5 hybrids tested
[b] : Acid detergent fiber.
[c] : Neutral detergent fiber.

In summary, within a given field trial location, either France or Italy, the compositional data was comparable across all hybrids. This is evidenced by the overlap in the range of values for each characteristic. The NIR and AOAC results are consistent, validating the utility of either method. These data demonstrate that under similar growing conditions, the composition of the grain and forage of the Insect-Protected maize hybrids are equivalent to the control hybrids grown commercially.

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STATEMENT OF CONFIDENTIALITY

Some of the information contained in the application has been designated as Confidential Business Information. This information, in Appendix II, contains the sequence of plasmid PV-ZMBK07.

The disclosure of this information would result in substantial harmful effect to Monsanto. If it became available to Monsanto's competitors who are working on insect resistance in crops, it would be extremely useful to them in competing with Monsanto and allow them to take shortcuts in their research and registration by copying our work and thus unfairly competing or causing financial loss and other harm to Monsanto.

All of this information is and has been maintained confidentially by Monsanto and security measures are taken to preserve its secrecy. This confidential business information was developed by Monsanto at its own expense, has not been released to anyone not under a secrecy agreement to Monsanto and has not been published. We request that this information not be released and protected.

Appendix I

Molecular Analyses of Insect-Protected Maize Line MON 810

Molecular Analysis of Insect Protected Maize Line MON 810

Janice Kania, Pamela Keck, Elaine Levine and Patricia Sanders
Monsanto Technical Report, MSL-14382

I. SUMMARY

This report describes the molecular analysis of the integrated DNA in Insect Protected maize line MON 810. Specifically, the insert number (number of integration sites within the maize genome) and the number and integrity of the inserted genes were determined. Maize line MON 810 was produced by particle acceleration technology using a DNA solution containing two plasmids, PV-ZMBK07 and PV-ZMGT10. The maize transformation vectors used to produce maize line MON 810 contain genes encoding 1) *cryIA(b)* gene (Höfte and Whiteley, 1989); 2) CP4 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4 EPSPS) (Padgette *et al.*, 1993); 3) glyphosate oxidoreductase (*gox*) (Padgette *et al.*, 1994); and 4) the *nptII* gene, under the control of a bacterial-specific promoter. Molecular analysis of maize line MON 810 established that the line only contains the *cryIA(b)* gene from plasmid PV-ZMBK07. The line does not contain the CP4 EPSPS, *gox*, or *nptII* genes. There is no evidence that any of the DNA contained in plasmid PV-ZMGT10 was inserted. Maize line MON 810 contains one integrated DNA, contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, maize hsp70 intron and the *cryIA(b)* gene.

Genetic Element	Maize Line MON 810
cryIA(b) gene	present
CP4 EPSPS gene	not present
gox gene	not present
nptII/*ori-p*UC	not present

ABBREVIATIONS

B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
bp	base pair (DNA)
cryIA(b)	Class I (Lepidoptera-specific) crystal protein gene
CP4 EPSPS	the gene or the protein, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
CTAB	cetyltrimethylammonium bromide
CTP-1	chloroplast transit peptide from *Arabadopsis thaliana* RUBISCO
CTP-2	chloroplast transit peptide from *Arabadopsis thaliana* EPSPS
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
E35S	Cauliflower mosaic virus (CaMV) 35S promoter with enhancer sequence
ECB	European corn borer
EDTA	ethylenediaminetetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GOX	glyphosate oxidoreductase protein
gox	gene for glyphosate oxidoreductase
B.t.k. HD-1	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1
HSP70	intron sequence from heat-shock protein 70 of maize
Kb	kilobase (DNA)
kD	kilodaltons
lacZ-alpha	gene for the alpha region of ß-galactosidase
µg	microgram
mL	milliliter
mM	millimolar
M	molar (moles/liter)
MW	molecular weight
NOS 3'	3' transcriptional termination sequence from nopaline synthase
nptII	gene for neomycin phosphotransferase II
NPTII	neomycin phosphotransferase II
ori-pUC	bacterial origin of replication from the pUC plasmid
PCR	polymerase chain reaction
pg	picogram
RNase	ribonuclease
SDS	sodium dodecylsulfate
SSC	20X is 3 M sodium chloride, 0.3 M sodium citrate
SOP	standard operating procedure
subsp.	subspecies
Tris	tris(hydroxymethyl)-aminomethane
TE buffer	10 mM Tris, 1 mM EDTA

II. INTRODUCTION

Maize plants (*Zea mays* L.) have been genetically modified to be protected against the European corn borer (ECB, *Ostrinia nubilalis*), a major insect pest of maize. Protection was accomplished by insertion of the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* HD-1 [*B.t.k.* HD-1], (Höfte and Whiteley, 1989) which encodes a protein insecticidal to Lepidoptera larvae but safe to mammals, fish, birds and non-target insects.

The maize genotype High Type II (Hi-II, Armstrong *et al.*, 1991) was transformed using particle acceleration. The plasmid maps for the two vectors used in the maize transformation DNA solution are shown in Figure 1 along with the restriction sites used for Southern blot analyses. One plasmid, PV-ZMBK07, contained the *cryIA(b)* gene and the second plasmid, PV-ZMGT10, contained two genes used for plant selection on glyphosate, a naturally glyphosate tolerant CP4 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS) and a glyphosate degrading enzyme, glyphosate oxidoreductase (GOX) (Padgette *et al.*, 1993, Barry *et al.*, 1992 and 1994). Both plasmids contained the *nptII* gene encoding neomycin phosphotransferase II (Beck *et al.*, 1982), under the control of its own bacterial promoter, to facilitate selection in bacteria, as well as the ori region required for bacterial replication.

The purpose of this investigation was to characterize the integrated DNA in the maize line MON 810. The DNA was evaluated using Southern blot analysis for the number of insertion events, and for the number and integrity of the genes contained within the insert.

III. MATERIALS & METHODS

A. Maize DNAs. The insect protected maize line, MON 810, was crossed to contain a [Mo17 X (Hi-II X B73)] background. The seed lot used for this analysis was designated MON 81000. The control line, MON 818 [Mo17 X (Hi-II X B73)], does not contain the *cryIA(b)*, CP4 EPSPS or *gox* genes. The seed lot used for this analysis was designated MON 81800. MON 810 and MON 818 DNAs were isolated from young leaf tissue collected from plants grown in a Monsanto greenhouse from seed harvested in the 1994 GLP field trials (Sanders *et al.*, 1995).

B. Reference substance. The reference substances for this investigation included the plasmids PV-ZMBK07 and PV-ZMGT10 (Figure 1). These plasmids or other *cryIA(b)* containing plasmids and DNA from the control line were digested with the same enzymes and either mixed or run separately. The mixed DNA samples provided an accurate size marker for the expected size fragments for each plasmid. The plasmid DNAs also served as positive hybridization controls. Additionally, molecular weight (MW) markers from Boehringer Mannheim (molecular weight markers II and IX, catalog #236 250 and catalog #1449 460, respectively) were used for size estimations. The two MW markers were mixed together, run on the gel and holes poked into the bands of the markers. After transfer, the holes were marked with dots of

water-proof ink, and later with ^{35}S ink, effectively transferring correct size standards. The lane containing the molecular weight marker dots was not scanned in the figures.

C. Method. The analytical procedure used for this investigation was Southern blot analysis, a common tool used for molecular characterizations (Southern, 1975). The reagents used during the course of this investigation are described in the SOPs for restriction digestion (SOP BtC-PRO-010), agarose gel electrophoresis (SOP DRT-PRO-003) and Southern blot analysis (SOP GEN-PRO-025).

D. DNA isolation. Young leaf tissue was removed from the plants and placed on dry ice during collection and stored at approximately -80°C prior to use. Approximately 1 g of frozen tissue was ground with a mortar and pestle and liquid nitrogen. The leaf powder was transferred to a polypropylene tube and approximately 6 mL of CTAB buffer [2.6% (w:w) sorbitol, 0.22 M Tris pH 8, 21 mM EDTA, 0.8 M NaCl, 22 mM CTAB, and 1% N-laurylsarcosine], prewarmed to approximately 65°C, was added to the ground frozen tissue. The samples were incubated at approximately 60°C for approximately 30 minutes and frequently inverted. The mixture was separated by centrifugation and the supernatant was extracted with phenol:chloroform (1:1, v/v). The supernatant was removed and an equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added. The supernatant was precipitated with approximately 0.6 volumes of isopropanol, inverted gently and stored at approximately 4°C overnight. The pellet was washed with 70% ethanol pre-chilled to approximately -20°C, vacuum dried, and resuspended in TE, pH 8. The DNA was treated with RNase, quantitated, and stored at approximately 4°C.

E. DNA quantitation and restriction enzyme digestion. The amount of DNA in samples was quantitated using a Hoefer TKO fluorometer (San Francisco, CA) (SOP GS-EQP-024). Approximately 10-15 µg of the isolated genomic DNA from the test and control lines were used for the restriction enzyme digests. Digests were performed according to SOP BtC-PRO-010. All restriction enzymes were purchased from Boehringer Mannheim (Indianapolis, IN).

F. DNA probe preparation. Plasmid DNAs (PV-ZMBK07 and PV-ZMGT10) were isolated from an *E. coli* culture. Probes homologous to the *cryIA(b),* CP4 EPSPS, *gox*, *nptII*, and *ori-p*UC genetic regions were prepared either by polymerase chain reaction (PCR) or isolated from plasmid DNA using the appropriate restriction enzyme digestion and agarose gel separation and purification (Gene Clean Kit, Bio 101, Vista, CA or QIAquick Gel Extraction Kit, Qiagen, Chatsworth, CA). All probes were radioactively labelled with ^{32}P using the random priming method (Prime-It® II Random Priming Kit, Stratagene, La Jolla, CA) according to SOP-PRO-073.

G. Southern blot analysis. Southern blot analyses were performed according to SOP GEN-PRO-025. The samples of DNA treated with restriction enzymes were separated, based on size, using 1.0% agarose gel

electrophoresis according to SOP DRT-PRO-003. The gels were usually electrophoresed for ≈13 hours at ≈35 volts and then for ≈2 hours at ≈60 volts. The DNA from the agarose gels was transferred to Hybond-N™ (Amersham, Arlington Heights, IL) nylon membrane using a Turboblotter (Schleicher & Schuell, Keene, NH). The DNA was allowed to transfer for approximately 16 hours (using 20X SSC as the transfer buffer) and covalently cross-linked to the membrane with a Stratalinker™ (Stratagene, La Jolla, CA). The blots were prehybridized an average of 4 hours in an aqueous solution of 0.5 M $Na_2HPO_4 \cdot 7H_2O$, 7% SDS. Hybridization with the radiolabeled probe was prepared in fresh prehybridization solution and typically for 14 to 16 hours at approximately 65°C. Membranes were washed in an aqueous solution of 40 mM $Na_2HPO_4 \cdot 7H_2O$, 5% SDS for two approximately 30 minute periods at approximately 65°C. Typical film exposure times were 1-3 days.

IV. RESULTS AND DISCUSSION

A. Southern blot results

Plasmid PV-ZMBK07 contained the *cryIA(b)* gene and plasmid PV-ZMGT10 contained the CP4 EPSPS and *gox* genes. The maps of the two plasmid vectors, along with the locations of the restriction sites utilized for Southern analyses, are presented in Figure 1.

The DNAs from MON 818 and MON 810 were digested with a variety of restriction enzymes and subjected to Southern blot hybridization analyses to characterize the DNA that was transferred during the particle acceleration into the maize genome. Specifically, the insert number (number of integration sites within the maize genome), and the copy number and integrity of each gene were examined.

B. Insert Number

NdeI digestion results. The purpose of the NdeI digests was to determine the number of plasmid DNA inserts in the maize line MON 810. The plasmids PV-ZMBK07 and PV-ZMGT10 do not contain a restriction site for NdeI. Thus this enzyme effectively cleaves outside any inserted DNA, releasing a fragment containing the inserted DNA and adjacent genomic DNA. MON 818 control DNA and MON 810 DNA were digested with NdeI and probed with plasmid PV-ZMBK07 DNA. The results are shown in Figure 2. MON 818 DNA, (lane 1), produced one very light, diffused band of approximately 21.0 Kb which is a background band since it is present in both the control MON 818 DNA and the MON 810 DNA. MON 810 DNA produced one band, approximately 5.5 Kb in size (lane 2). This result established that insect protected maize line MON 810 contains one fragment of integrated DNA. The size of the inserted DNA plus adjacent genomic DNA up to the NdeI restriction sites is approximately 5.5 Kb in size.

C. Insert Composition

1. *cryIA(b)* gene integrity. MON 818 and MON 810 DNAs were digested with NcoI/EcoRI to release the *cryIA(b)* gene and the Southern blot probed with the *cryIA(b)* gene. The results are shown in Figure 3, lanes 1-3. The

positive hybridization control (lane 1) produced one 3.46 Kb fragment which corresponds to the expected size of the *cryIA(b)* gene (refer to the plasmid maps in Fig. 1). Due to the plasmid DNA not being mixed with genomic control DNA the band appears of a larger molecular weight than its true molecular weight. The MON 818 DNA (lane 2) does not produce any bands, as expected for the control line. The MON 810 DNA (lane 3) contains one band, approximately 3.1 Kb. The NcoI/EcoRI digests, probed with the *cryIA(b)* gene, identified one *cryIA(b)* gene, approximately 3.1 Kb, sufficient to encode an insecticidally active CryIA(b) protein (Höfte *et al.*, 1986) in the insect protected maize line MON 810.

Western blot analyses were performed to evaluate the size of the CryIA(b) protein expressed in line MON 810 compared to *E. coli* produced protein standards. This analysis is described in detail in Attachment 1. As is commonly observed in the western analysis of *B.t.k.* proteins (Attachment 1, Lee and Bailey, 1995), multiple protein products were observed for all the insect protected maize lines analyzed (Attachment 1, Fig. 1). Maize line MON 810 produced the expected trypsin resistant core protein (≈63 kD) (Attachment 1, Fig. 2, lane 10). Based on the western blot data and efficacy of maize line MON 810, the *cryIA(b)* gene present produces an insecticidal CryIA(b) protein which provides effective, season long control of European Corn Borer.

2. CP4 EPSPS gene integrity. Plasmid DNAs (PV-ZMBK07 and PV-ZMGT10) and insect protected maize line MON 810 DNA were digested with NcoI/BamHI to release the CP4 EPSPS gene and the Southern blot probed with the CP4 EPSPS gene. The results are shown in Figure 4, lanes 1 and 2. Approximately 50 pg of a mixture of PV-ZMBK07 and PV-ZMGT10 DNA (lane 1) produced one band, approximately 3.1 Kb in size, which corresponds to the expected size CP4 EPSPS fragment, as predicted from the plasmid map (PV-ZMGT10 in Fig. 1). MON 810 DNA (lane 2) shows no hybridizing fragements to the CP4 EPSPS probe, establishing that insect protected maize line MON 810 does not contain the CP4 EPSPS gene. As expected, the CP4 EPSPS protein was not detected by ELISA in leaf, whole plant or grain tissues of MON 810 (Sanders *et al.*, 1995). Western blot analyses confirmed the absence of CP4 EPSPS protein in maize line MON 810, no bands were observed in the leaf extracts of MON 810 (Attachment 1, Fig. 3, lane 9).

3. *gox* gene integrity. Plasmid DNAs (PV-ZMBK07 and PV-ZMGT10) and insect protected maize line MON 810 DNA were digested with NcoI/BamHI to release the *gox* gene and the Southern blot probed with the *gox* gene. The results are shown in Figure 4, lanes 3 and 4. Approximately 50 pg of a mixture of PV-ZMBK07 and PV-ZMGT10 DNA (lane 3) produced one band, approximately 3.1 Kb, which corresponds to the expected size *gox* fragment, as predicted from the plasmid map (PV-ZMGT10 in Fig. 1). MON 810 DNA (lane 4) shows no hybridizing fragments to the *gox* probe, establishing that insect protected maize line MON 810 does not contain the *gox* gene. As expected, the *gox* protein was not detected by ELISA in leaf, whole plant or grain tissues of MON 810 (Sanders *et al.*, 1995). Western blot analyses confirmed the absence

of a gox protein in maize line MON 810, no bands were observed in the leaf extracts of MON 810 (Attachment 1, Fig. 4, lane8).

4. Backbone integrity. Plasmids PV-ZMBK07 and PV-ZMGT10 (mixed with control DNA, MON 818), control line MON 818 and insect protected maize line MON 810 DNAs were digested with NcoI/EcoRI to release the nptII/ori-pUC backbone. The digested DNAs were run on a Southern blot and the blot was probed with the nptII/ori-pUC sequences. The results are shown in Figure 5 (lanes 1-6):
- The PV-ZMBK07 plasmid DNA produced two bands of 2.5 Kb and 1.8 Kb (lane 1). The 2.5 Kb and 1.8 bands correspond to the expected size fragments of the backbone from vector PV-ZMBK07 (Fig. 1).
- The PV-ZMGT10 plasmid produced two bands of 1.5 and 3.0 Kb (lane 3) which are the predicted backbone fragments for the vector (Fig. 1).
- The MON 818 DNA alone (lane 5) does not produce any bands, as expected from a non-modified control line.
- MON 810 DNA (lane 6) shows no bands, establishing that the backbone sequences were not integrated in insected protected maize line MON 810.

The Southern blot was stripped and reprobed with the maize *hsp70* genetic region (Fig. 6). There are two endogenous maize *hsp70* bands (1.2 and 1.5 Kb) which appear in all lanes containing maize genomic DNA (lanes 1, 3, 5 and 6).
- The PV-ZMBK07 DNA (lane 1) produced an additional band, 2.5 Kb, as predicted from the plasmid map (Fig. 1). PV-ZMGT10 DNA (lane 3) contains two additional bands, of 2.0 and 3.0 Kb as predicted from the plasmid map (Fig. 1).
- The MON 818 DNA alone (lane 5) contains only the endogenous maize *hsp70* bands (1.2 and 1.5 Kb), as expected for the unmodified control line.
- MON 810 DNA (lane 6) contains the two endogenous maize *hsp70* bands, and an 8.0 Kb band which contains the maize *hsp70* intron associated with the *cryIA(b)* gene.
The maize *hsp70* results demonstrate that a single copy of a gene, if present, could be detected by Southern blot analysis.

V. CONCLUSIONS

The insect protected maize line MON 810 was produced by particle acceleration technology with a DNA solution that contained the *cryIA(b),* CP4 EPSPS, *gox* and *nptII* genes. Maize line MON 810 contains one integrated DNA contained on a 5.5 Kb NdeI fragment, which contains the E35S promoter, maize hsp70 intron and the *cryIA(b)* gene. Western blot analyses established the expected trypsin resistant core of the CryIA(b) protein was produced. Insect protected maize line MON 810 does not contain a CP4 EPSPS gene, a *gox* gene or *nptII/ori-pUC* sequences. The continued efficacy of maize line MON 810 confirms that an insecticidally active CryIA(b) protein is produced which provides season long control of European Corn Borer.

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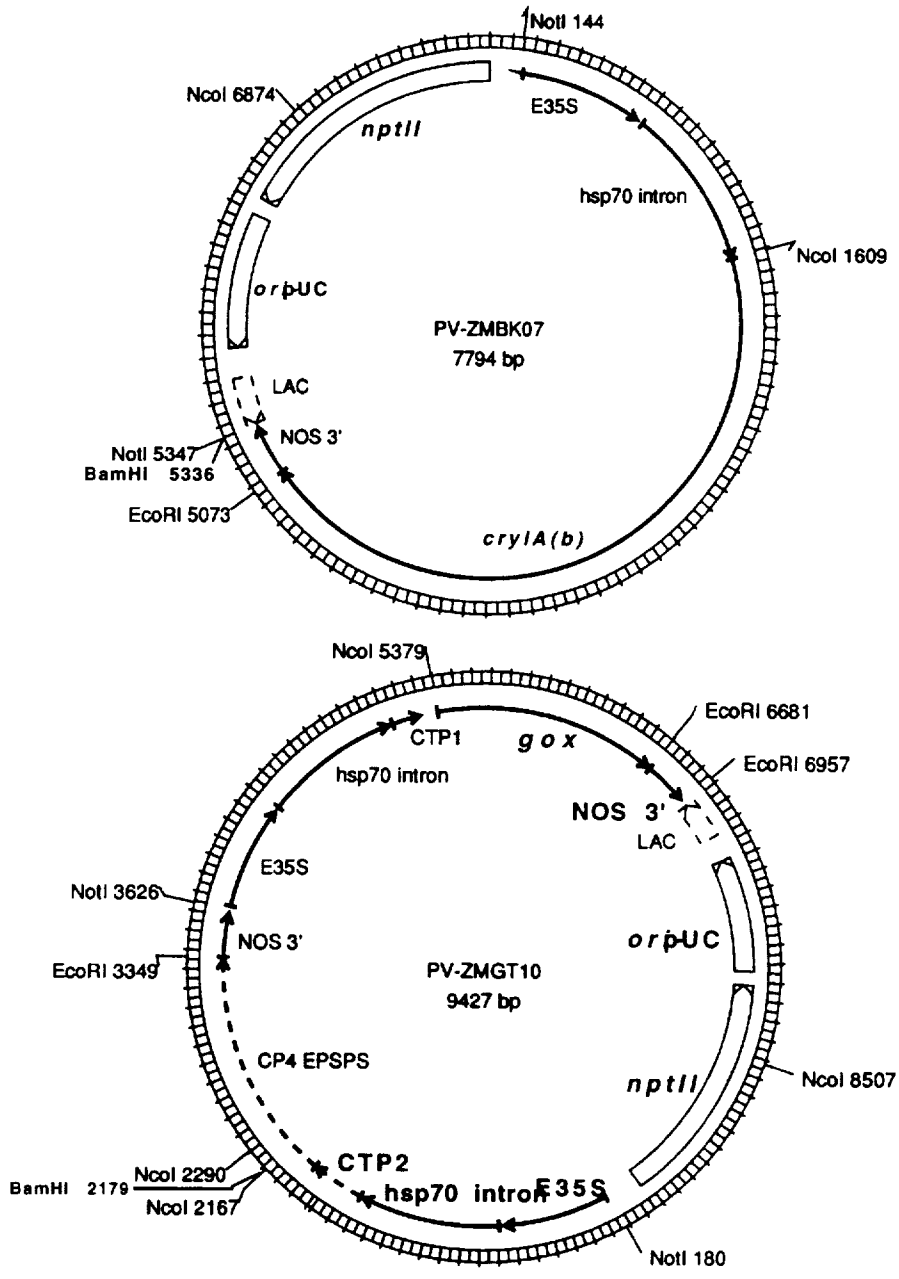

Figure 1. Plasmid maps of PV-ZMBK07 and PV-ZMGT10. Restriction sites, and their locations in base pairs, used during Southern analyses are shown.

Figure 2. Southern blot analysis of maize line MON 810 DNA: insert number analysis

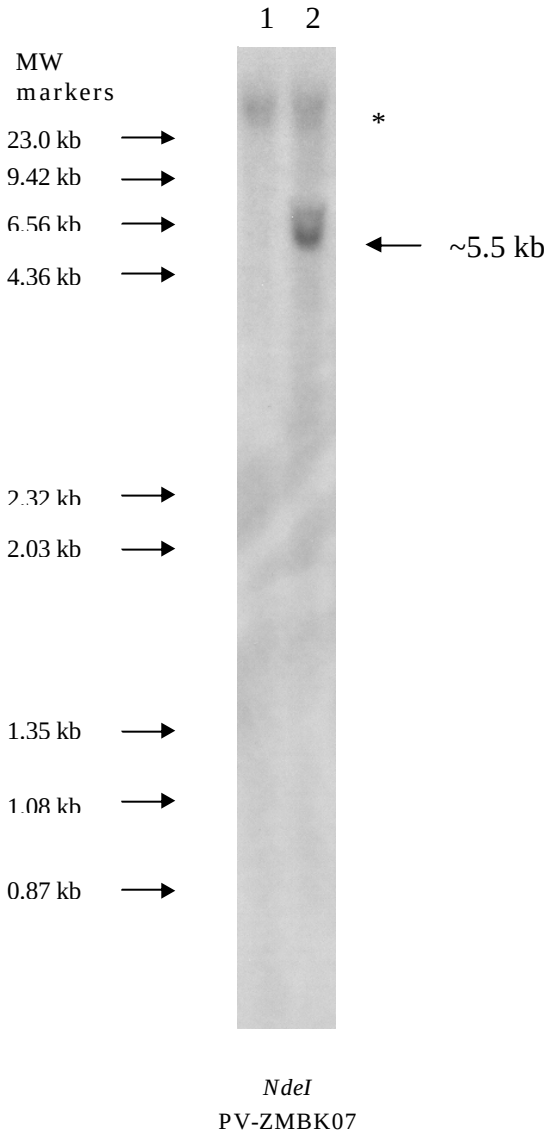

Figure 2. Southern blot analysis of maize line MON 810 DNA: Lanes 1 and 2 contain the following DNAs digested with *NdeI* and probed with PV-ZMBK07: lane 1, MON 818 DNA; lane 2, MON 810 DNA.

→ Symbol denotes sizes obtained from MW markers.
~ Symbol denotes a band size approximated from MW marker and plasmid digests.
* Symbol denotes background bands.

Figure 3. Southern blot analysis of maize line MON 810 DNA: *cryIA(b)* gene analysis

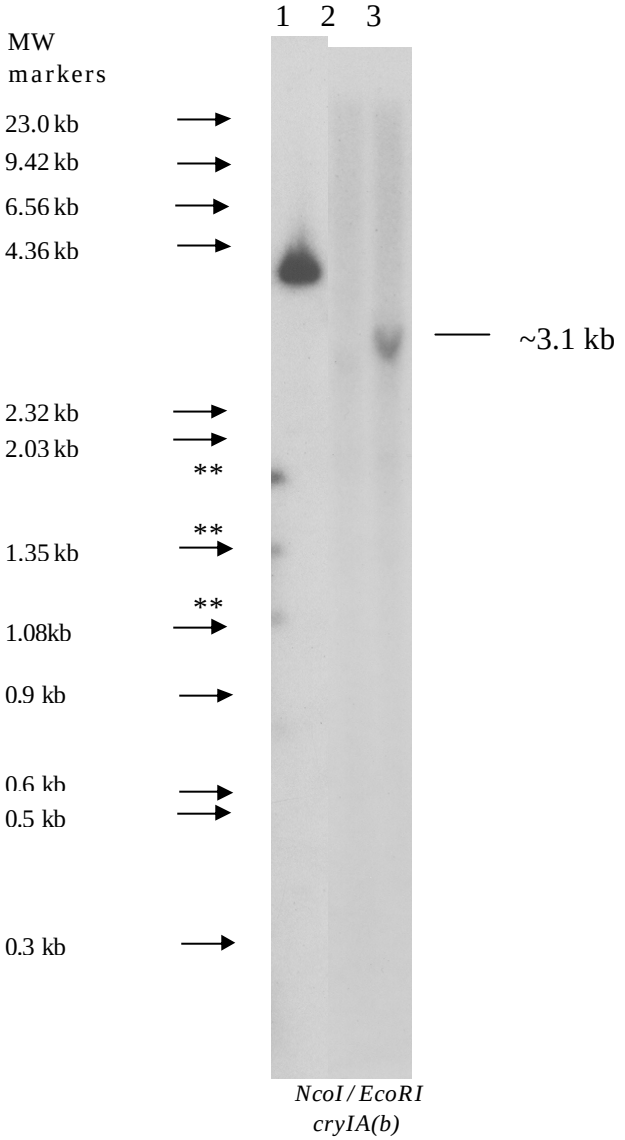

Figure 3. Southern blot analysis of maize line MON 810 DNA: Lanes 1-3 contain the following DNAs digested with *NcoI / EcoRI* and probed with the *cryIA(b)* gene: lane1, ≈50 pg of plasmid PV-ZMBK07; lane 2, MON 818 DNA; lane 3, MON 810 DNA.

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.

—— Symbol denotes sizes obtained from plasmid digests.

~ Symbol denotes a band size approximated from MW marker and plasmid digests.

** Symbol denotes an area of hybridization in an adjacent lane which only appears to be in lane 1, due to the contents of the lanes migrating at an angle in this portion of the gel.

Figure 4. Southern blot analysis of maize line MON 810 DNA: *cp4 epsps* and *gox* gene analysis

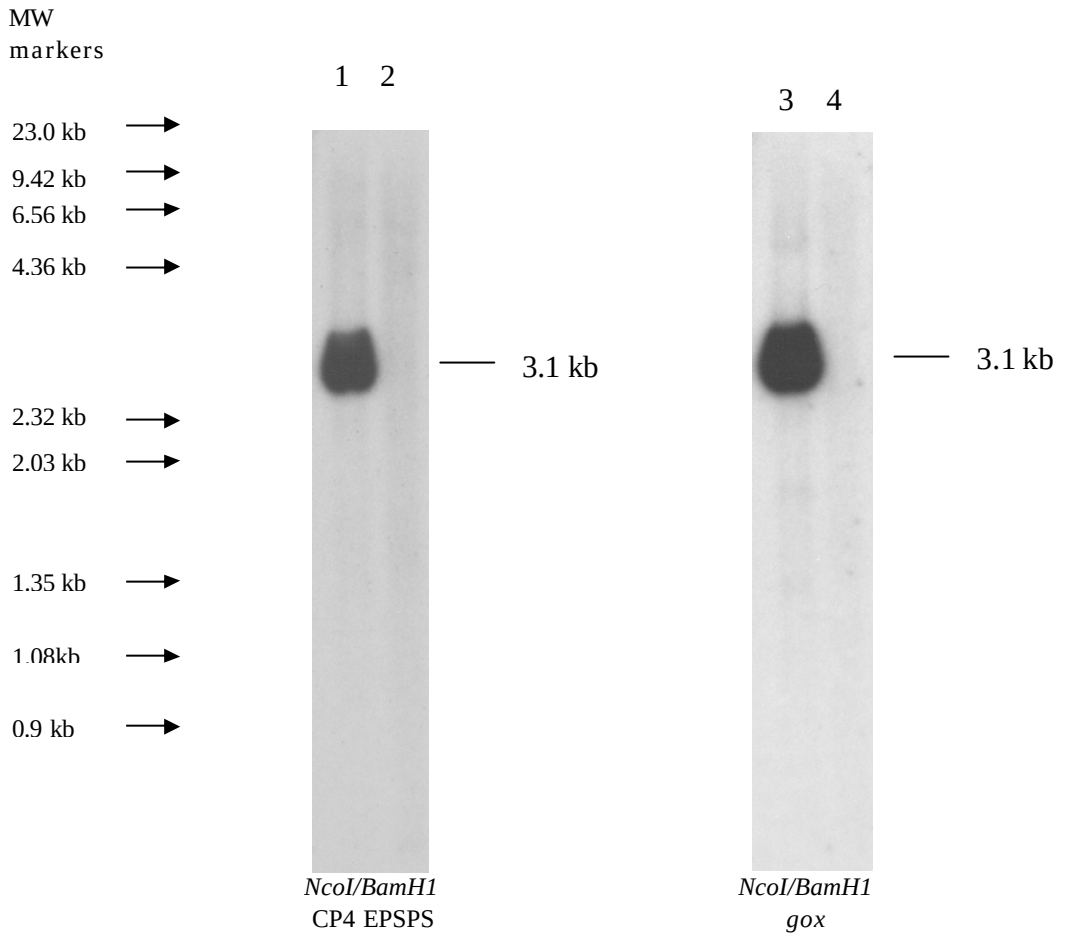

Figure 4. Southern blot analysis of maize line MON 810 DNA: Lanes 1-4 contain the following DNAs digested with *NcoI/BamH1*: lane 1 and 3, ≈50 pg of plasmids PV-ZMGT10 and PV-ZMBK07; lane 2 and 4, MON 810 DNA. Lanes 3 and 4 were hybridized with the *gox* gene.

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.

Figure 5. Southern blot analysis of maize line MON 810 DNA: *nptII/ori-pUC* gene analysis

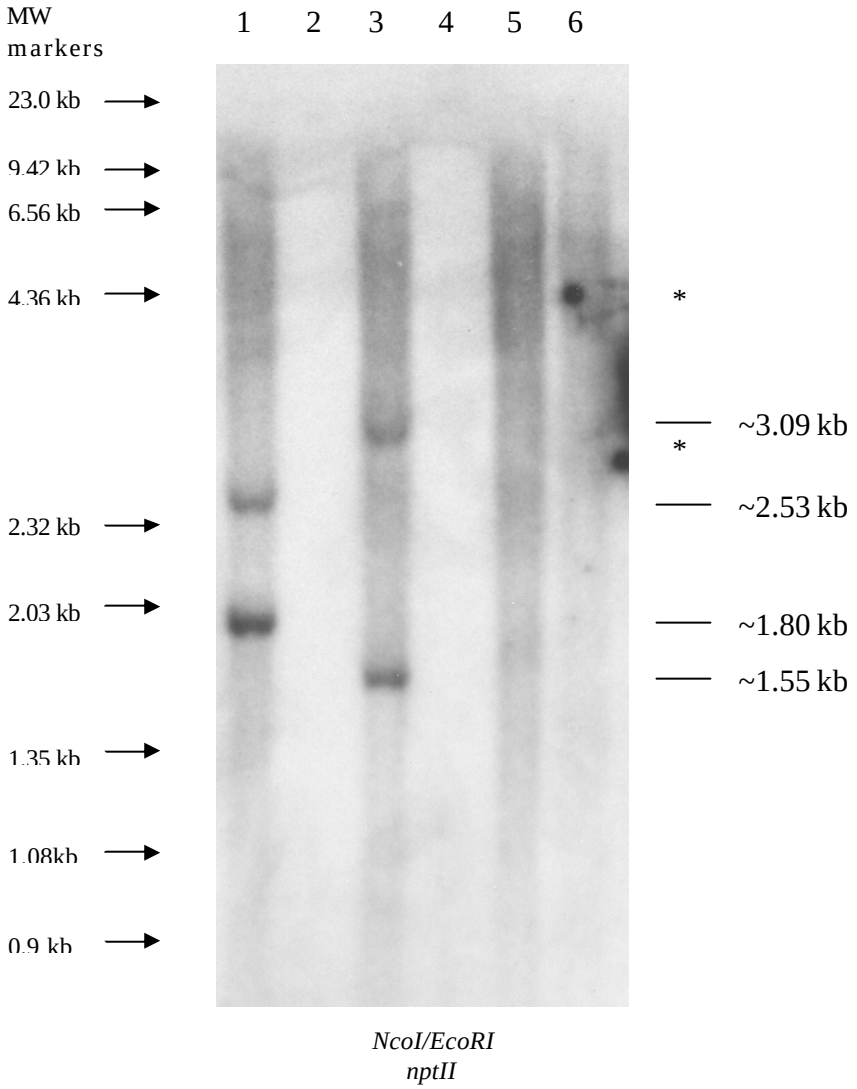

Figure 5. Southern blot analysis of maize line MON 810 DNA: Lanes 2 and 4 are empty. Lanes 1, 3, 5 and 6 contain the following DNAs digested with *NcoI/EcoRI* and probed with *nptII/ori-p*UC: lane1, MON 818 DNA with ≈50 pg of PV-ZMBK07; lane 3, MON 818 DNA with ≈50 pg of PV-ZMGT10; lane 5, MON 818 DNA; lane 6, MON 810 DNA.

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
~ Symbol denotes a band size approximated from MW marker and plasmid digests.
* Symbol denotes an area of non-specific hybridization.

Figure 6. Southern blot analysis of maize line MON 810 DNA: maize *hsp70* analysis

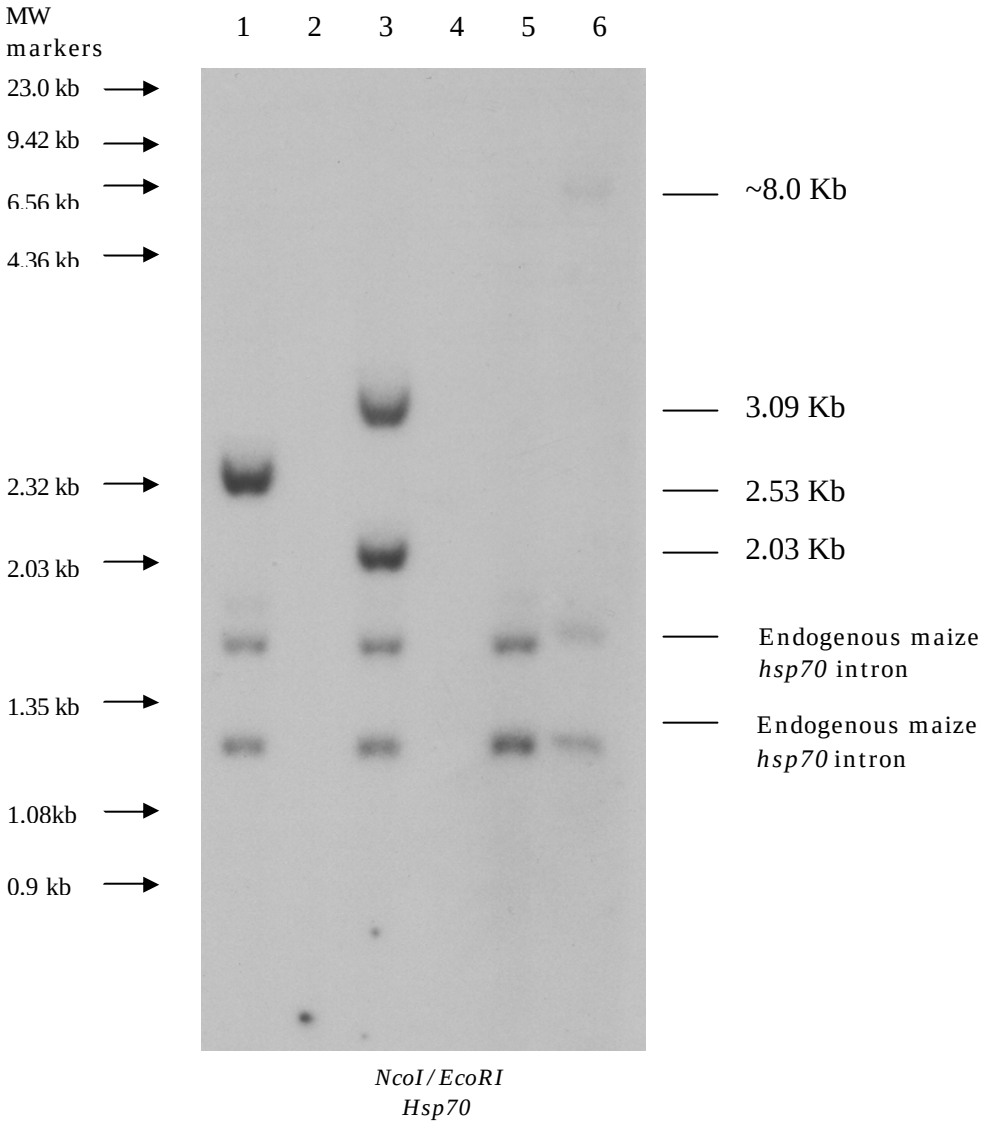

Figure 6. Southern blot analysis of maize line MON 810 DNA: Lanes 2 and 4 are empty. Lanes 1, 3, 5 and 6 contain the following DNAs digested with *NcoI/EcoRI* and probed with maize *hsp70* fragment: lane 1, MON 818 DNA with ≈50 pg of PV-ZMBK07; lane 3, MON 818 DNA with ≈50 pg of PV-ZMGT10; lane 5, MON 818 DNA; lane 6, MON 810 DNA.

→ Symbol denotes sizes obtained from MW markers on ethidium stained gel.
—— Symbol denotes sizes obtained from plasmid digests.
~ Symbol denotes a band size approximated from MW marker and plasmid digests.

ATTACHMENT 1

WESTERN BLOT ANALYSES OF INSECT PROTECTED AND INSECT PROTECTED / ROUNDUP READY™ CORN LINES

by Thomas Lee and Michelle Bailey

I. SUMMARY

Plasmid DNAs can break during the process of particle acceleration, resulting in the integration of partial genes into the genomic DNA. Therefore, western blot analyses for the protein products of the three integrated genes, *cryIA(b)*, CP4 EPSPS, and *gox*, were conducted using tissue derived from five Insect Protected (IPC) and two Insect Protected/Roundup Ready™ (IPC/RR) lines. Western blot analyses enabled evaluation of the presence and sizes of the expressed proteins using antibodies specific to the protein under examination. Comparisons to protein standards (isolated from *E. coli*) and tissue extracts from other Insect Protected corn lines containing the same genes were conducted to evaluate if the produced proteins were of the expected size and if any unexpected protein products were produced.

In summary, the CryIA(b), CP4 EPSPS, and GOX proteins in the Insect Protected (IPC) and Insect Protected/Roundup Ready™ (IPC/RR) corn lines show immunoreactive products of the expected sizes (if present) when compared with purified protein standards. The immunoreactive products observed were similar among the seven insect protected corn lines which contain the *cryIA(b)*, CP4 EPSPS and *gox* genes.

ABBREVIATIONS

B.t.k.	*Bacillus thuringiensis* subsp. *kurstaki*
B.t.k. HD-1	*Bacillus thuringiensis* subsp. *kurstaki* strain HD-1
$CaCl_2$	Calcium chloride
CHAPS	3[(3-Cholamidopropyl)dimethylammonio]1-propanesulfonate
cryIA(b)	Class I (Lepidoptera-specific) crystal protein gene
CP4 EPSPS	the gene or the protein, 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4
E. coli	*Escherichia coli*
ECB	European corn borer
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis-(beta-amino ethyl ether) tetraacetic acid
EPSPS	5-enolpyruvylshikimate-3-phosphate synthase
GOX	glyphosate oxidoreductase protein
gox	gene for glyphosate oxidoreductase
h	hour
kD	kilodaltons
lacZ-alpha	gene for the alpha region of ß-galactosidase
µg	microgram
µl	microliter
min	minute
mL	milliliter
mM	millimolar
M	molar (moles/liter)
MW	molecular weight
NBT/BCIP	Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate
NOS 3'	3' transcriptional termination sequence from nopaline synthase
nptII	gene for neomycin phosphotransferase II
NPTII	neomycin phosphotransferase II
*ori-p*UC	bacterial origin of replication from the pUC plasmid
pg	picogram
PMSF	phenylmethylsulfonyl flouride
PVDF	polyvinylidene difluoride
RPM	revolutions per minute
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SSC	20X is 3 M sodium chloride, 0.3 M sodium citrate
SOP	standard operating procedure
subsp.	subspecies
w/v	weight per volume

II. INTRODUCTION

Corn plants (*Zea mays* L.) have been genetically modified to control the European corn borer (ECB, *Ostrinia nubilalis*), a major corn pest. Resistance was accomplished by insertion of the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* [*B.t.k.* HD-1] (Höfte and Whiteley, 1989) which encodes for the production of a protein insecticidal to Lepidoptera larvae. In addition to the gene for CryIA(b) protein, genes encoding 5-enolpyruvylshikimate-3-phosphate synthase (CP4 EPSPS, Padgette *et al.*, 1993) and glyphosate oxidoreductase (GOX, Padgette *et al.*, 1994) were also present in the DNA solution used during corn transformation to enable selection of cells in tissue culture, that may contain the gene for CryIA(b) protein.

During the process of particle acceleration, the plasmid DNA can break resulting in the integration of plasmid fragments into the genomic DNA. Therefore, western blot analyses were performed to identify the protein products (CryIA(b), CP4 EPSPS, and GOX) of the three integrated genes, using leaf tissue of the IPC and IPC/RR lines. Western blot analyses enabled evaluation of the presence and sizes of the expressed proteins using antibodies specific to the protein under examination. Comparisons to protein standards (isolated from *E. coli*) and tissue extracts from other Insect Protected corn lines containing the same genes were made to evaluate if the produced proteins were of the expected size and if any unexpected protein products were produced.

III. MATERIALS AND METHODS

A. Western Blot Analysis for CryIA(b)

Preparation of protein extracts from corn leaf tissue for the full length CryIA(b) protein assessment. Protein extracts of leaf tissue samples from the test corn lines (MON 801, MON 802, MON 805, MON 809, MON 810, MON 813, and MON 814) and parental control corn lines (MON 818 and MON 819) were prepared as follows for western blot analysis. Leaf tissue samples were initially ground to a fine frozen powder in the presence of liquid nitrogen. The powdered tissue samples (approximately 1.0- 1.7 g) were homogenized in a buffer solution containing 100 mM carbonate/bicarbonate buffer, pH 10.01, 10 mM 2-mercaptoethanol, 2.5 mM EDTA, 2.5 mM EGTA, 1.0 mM benzamidine-HCl, 0.5 mM PMSF, 1 µg/mL pepstatin A, 40 µg/mL bestatin, 1.0 mM CHAPS, and 10% (v/v) glycerol of a tissue:buffer ratio of 1 g of tissue to 5 mL of buffer. Homogenization was accomplished using a hand-held homogenizer (Tissue Tearor™, Model 985-370 type 2, Biospec Products, Inc., Racine, WI) for approximately 1 min on speed=5. Cell debris was removed by centrifugation at ≈20,000 x g for 15 min. A 2.5 mL aliquot of each supernatant was desalted on a PD-10 column (Pharmacia Biotech AB, Prod. No. 17-0851-01, Uppsala Sweden). Each desalted extract was concentrated ≈4-5 fold by centrifugation at ≈5000 RPM for 4.5 hours using Centricon 30 microconcentrators (Amicon, Prod. No. 4209, Beverly, MA). A 100 µl aliquot of each extract was diluted with 100 µl of 2X Laemmli buffer (Laemmli, 1970) (SepraSol, lot # S13-080, Integrated Separation Systems, Natick, MA) and heated for approximately 5

min at approximately 100°C. A second aliquot (200 µl) was removed for trypsinization (described below). The remainder of the extract was stored at -80°C.

The protein concentration of the desalted and concentrated extracts was estimated by the method of Bradford (1976), according to SOP GG-PRO-015-01. Bovine serum albumin was used as a standard (lot # 5526218).

Preparation of protein extracts and western blot analysis from corn leaf tissue for the trypsin-resistant protein assessment. A 200 µl aliquot of each extract (described above) was removed for trypsinization. Trypsinization of the clarified supernatant aliquots was initiated by the addition of 7 µl of a stock trypsin solution (5 mg/ mL bovine pancreatic, TPCK-treated, Sigma, St. Louis, Prod. No. T8642; 50 mM Tris-HCl, pH 8.0, 20 mM $CaCl_2$). The samples were incubated overnight at approximately 4°C with constant rocking on a Nutator (Clay Adams Co., Model No. 1105, Parsippany, NJ) rotating table. Trypsinization was quenched after approximately 18 h by the addition of 1.25 µl of a 200 mM stock solution of PMSF (Sigma, St. Louis, Prod. No. P-7626). A 50 µl aliquot of each quenched extract was diluted with 50 µl of 2X Laemmli buffer (SepraSol, lot # S11-102, Integrated Separation Systems, Natick, MA) and boiled for approximately 5 min.

SDS-PAGE. After heating, samples of the corn leaf protein extracts, the *E. coli*-produced trypsin-resistant core of the CryIA(b) protein and *E. coli*-produced CryIA(c) protein standards in Laemmli buffer (1970) were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) on 4-20% gradient gels according to SOP PB-EQP-005 using the mini gel system of NOVEX (San Diego, CA). Electrophoresis was conducted at constant voltage (approximately 200 V) for approximately 1 hour (until the dye front reached the bottom of the gel). Wide range color molecular weight markers (Sigma, St. Louis, Prod. No. 3437) were included in one lane of the gel to verify subsequent transfer to the polyvinylidene difluoride (PVDF) membrane.

Western blot analysis. Proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF (polyvinylidene difluoride) membrane (Immobilon P, Millipore Corp., Bedford, MA) according to SOP BtC-PRO-002-02. The protein blots were blocked for approximately one hour in 5% nonfat dry milk in TBST (100 mM TRIS, 10mM sodium borate, 150 mM sodium chloride, 0.05% (v/v) Tween-20) at room temperature. The protein blot was probed with an approximate 1:1000 dilution of rabbit antiserum F204 (bleed 10) raised against an *E. coli*-produced CryIA(b) trypsin-resistant core protein. Rabbit antibody bound to the blot was detected using an approximately 1:1000 dilution of donkey-anti-rabbit antibody conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA). Immunoreactive protein bands were visualized using the NBT/BCIP colorimetric substrate system (Promega, Madison, WI).

B. Western Blot Analysis for GOX and CP4 EPSPS

Sample preparation: Young leaf tissue samples of the test lines (MON 801, MON 802, MON 805, MON 809, MON 810, MON 813 and MON 814) and parental control lines (MON 818 and MON 819), were homogenized in a buffer solution containing 100 mM Tris, 100 mM sodium borate, 5 mM magnesium chloride pH 7.8, 0.5% (v/v) Tween-20, and 0.2% (w/v) L-ascorbic acid (added fresh). Each protein extract was centrifuged and the supernatant removed for analysis. Aliquots of the extracts were diluted 1:1 with 2X Laemmli buffer (SepraSol, Integrated Separations Systems, Natick, MA) and heated at approximately 100°C for approximately 5 minutes.

SDS-PAGE: After heating, the 1X Laemmli corn sample extracts and the appropriate *E. coli*-produced protein standards were analyzed by sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) using 4-20% gradient gels in accordance with SOP PB-EQP-005 using the mini gel system of Novex (San Diego, CA). Electrophoresis was conducted at constant voltage (200v) for approximately 1 hour until the dye front reached the bottom of the gel. Wide range color molecular weight markers (Sigma, St. Louis, Prod. No. 3437) were included in the gel to verify subsequent transfer to the PVDF membrane.

Western blot analysis: Proteins separated by SDS-PAGE were electrophoretically transferred to a PVDF membrane (Immobilon P, Millipore Corp., Bedford, MA) according to SOP BtC-PRO-002-02. The protein blots were blocked for approximately one hour in 5% nonfat dry milk in TBST (100 mM TRIS, 10mM sodium borate, 150 mM sodium chloride, 0.05% (v/v) Tween-20) at room temperature, followed by probing with a 1:1000 dilutions of goat antisera raised against the appropriate protein (DR1 bleed 7 for GOX, DR2 bleed 3 for CP4 EPSPS). Bound antibody was detected using a commercial donkey-anti-goat antibody conjugated to horseradish peroxidase (Jackson Immunoresearch, West Grove, PA). Immunoreactive bands were visualized using an enhanced chemiluminescent detection system (Amersham) followed by exposure to film (Hyperfilm, Amersham). In addition to plant protein extracts, *E. coli*-produced GOX or CP4 EPSPS reference standards were included for analysis.

IV. RESULTS AND DISCUSSION

A. CryIA(b) Protein

Non-trypsin treated extracts. The profile for the CryIA(b) protein from non-trypsin treated leaf extracts of seven Insect Protected corn lines was compared to a CryIA(c) full length (≈ 134 kD) standard and a CryIA(b) (≈ 63 kD) trypsin resistant core protein standard (Figure 1). The CryIA(c) full length standard was used because it is approximately the same size as the full length CryIA(b) protein (131 kD) and cross-reacts with the same antibody (Lee *et al.*, 1995). Two control lines, MON 818 and MON 819, were included to represent the genetic backgrounds of the seven test lines. Minor background bands were observed in extracts from both control lines, MON 818 and MON 819 (lanes 3 and 4). However, these background bands are observed in leaf extracts of all

corn lines (Insect Protected and controls).

A full length band at the predicted molecular weight (≈ 131 kD) was observed for lines MON 801, MON 802, MON 805, and MON 809 (lanes 5-8). Insect Protected corn line MON 810 contains a less than full length *cryIA(b)* gene and therefore does not show an 131 kD protein product (lane 9) (Kania *et al.*, 1995). As is commonly observed in the western analysis of *B.t.k.* protein (Lee *et al.*, 1995), multiple immunoreactive protein products were observed for the Insect Protected corn lines (lanes 5-11). There were no apparent differences in the size ranges of the less than full length protein products observed among the seven Insect Protected corn lines produced with the same full length *cryIA(b)* gene (lanes 5-11).

Trypsinized extracts. Minor immunoreactive bands were observed in extracts prepared from both control lines, MON 818 and MON 819 (Figure 2, lanes 3 and 4). However, these background bands are observed in leaf extracts of most corn lines (Insect Protected and controls). All seven IPC lines showed the common CryIA(b) trypsin-resistant core protein product at ≈ 63 kD (Figure 2, lanes 6 through 12), as previously reported (Lee and Bailey, 1995) following trypsin treatment. In all seven lines evaluated, no detectable qualitative differences in the trypsinized treated extracts were observed.

B. CP4 EPSPS protein

Extracts of young leaf tissue from the seven Insect Protected corn lines were compared to a CP4 EPSPS protein standard produced in *E. coli* (Figure 3). No band was observed with extracts from either control line, MON 818 and MON 819 (lanes 5 and 6). No CP4 EPSPS protein was detected for corn line MON 814 (lane 7). Protein extracts from young leaf tissues of Insect Protected corn lines MON 813, MON 809, MON 805, MON 802 and MON 801 showed the expected 47.6 kD CP4 EPSPS protein product when compared to the purified CP4 EPSPS protein standard (lanes 8, 10-14). The corn line MON 810 does not contain the CP4 EPSPS gene and therefore does not express the CP4 EPSPS protein (lane 9) (Kania *et al.*, 1995).

C. GOX protein

Young leaf tissue extracts from the seven Insect Protected corn lines were compared to a GOX protein standard produced in *E. coli* (Figure 4). Protein extracts from corn lines MON 802, MON 805, MON 813 and MON 814 (lanes 5, 6, 9 and 10) showed the expected 47 kD protein product when compared to the purified GOX protein standard (lanes 2, 14 and 15). The GOX protein was not detected in corn lines MON 801, MON 809 and MON 810 (lanes 4, 7 and 8). The GOX protein level in corn line MON 801 leaves is below the limit of detection for western blot analysis and enzyme linked immunosorbent assay (ELISA) (Sanders *et al.*, 1995). The corn line MON 810 does not contain the *gox* gene and therefore does not express the gox protein (Kania *et al.*, 1995). As expected, no bands were observed in extracts from the two control lines, MON 818 and MON 819 (Figure 4, lanes 11 and 12).

V. CONCLUSION

In summary, the CryIA(b), CP4 EPSPS, and GOX proteins in the Insect Protected corn lines show immunoreactive products of the expected sizes (when present) when compared with the respective purified protein standards. The immunoreactive products observed were similar among the seven Insect Protected corn lines which contain the *cryIA(b)*, CP4 EPSPS and *gox* genes.

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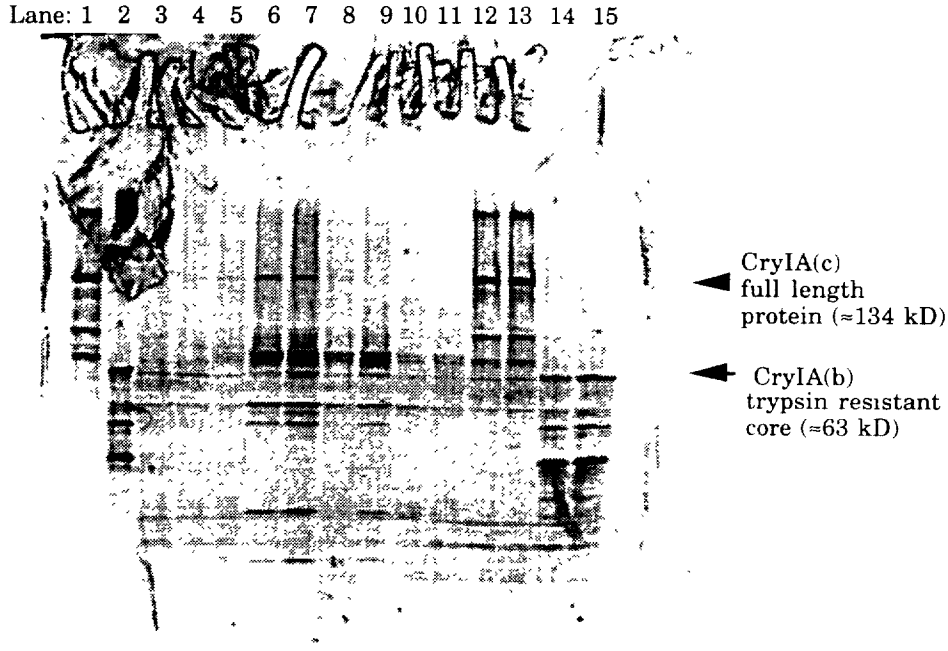
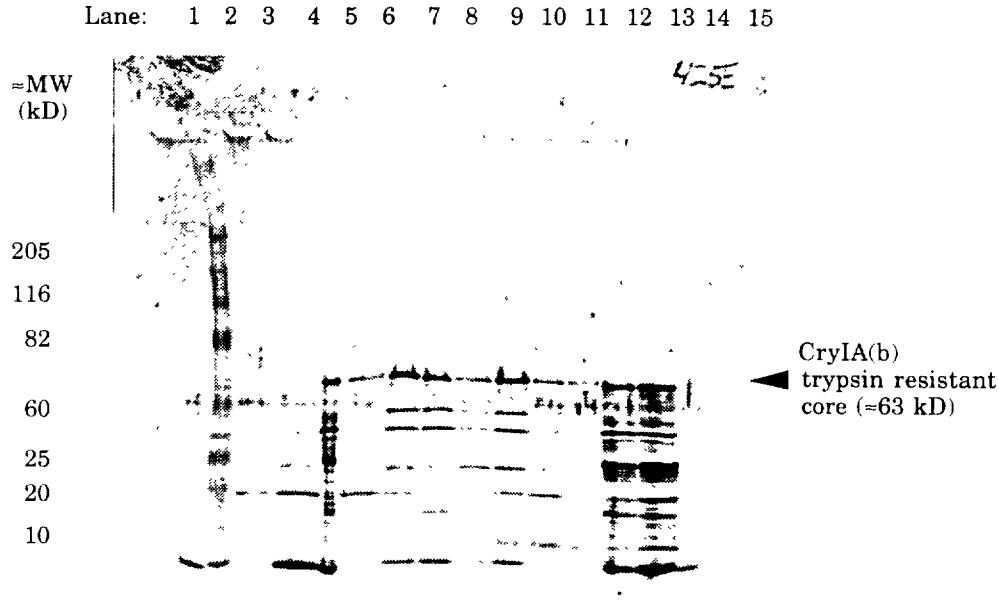
Figure 1. Western blot analysis of CryIA(b) proteins in corn tissue extracts.



Lane	Description
1	*E. coli*-produced CryIA(c) full length protein, ≈20 ng loaded
2	*E. coli*-produced CryIA(b) trypsin-resistant core, ≈20 ng loaded
3	MON 818 leaf protein extract, ≈28 µg loaded
4	MON 819 leaf protein extract, ≈24 µg loaded
5	MON 801 leaf protein extract, ≈39 µg loaded
6	MON 802 leaf protein extract, ≈25 µg loaded
7	MON 805 leaf protein extract, ≈32 µg loaded
8	MON 809 leaf protein extract, ≈26 µg loaded
9	MON 810 leaf protein extract, ≈26 µg loaded
10	MON 813 leaf protein extract, ≈27 µg loaded
11	MON 814 leaf protein extract, ≈20 µg loaded
12	*E. coli*-produced CryIA(c) full length protein, ≈20 ng spiked into ≈19 µg of MON 818 leaf protein extract
13	*E. coli*-produced CryIA(c) full length protein, ≈20 ng spiked into ≈16 µg of MON 819 leaf protein extract
14	*E. coli*-produced CryIA(b) trypsin-resistant core, ≈20 ng, spiked into ≈19 µg of MON 818 leaf protein extract
15	*E. coli*-produced CryIA(b) trypsin-resistant core, ≈20 ng, spiked into ≈16 µg of MON 819 leaf protein extract

Figure 2. Western blot analysis of trypsinized CryIA(b) proteins in corn tissue extracts.



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

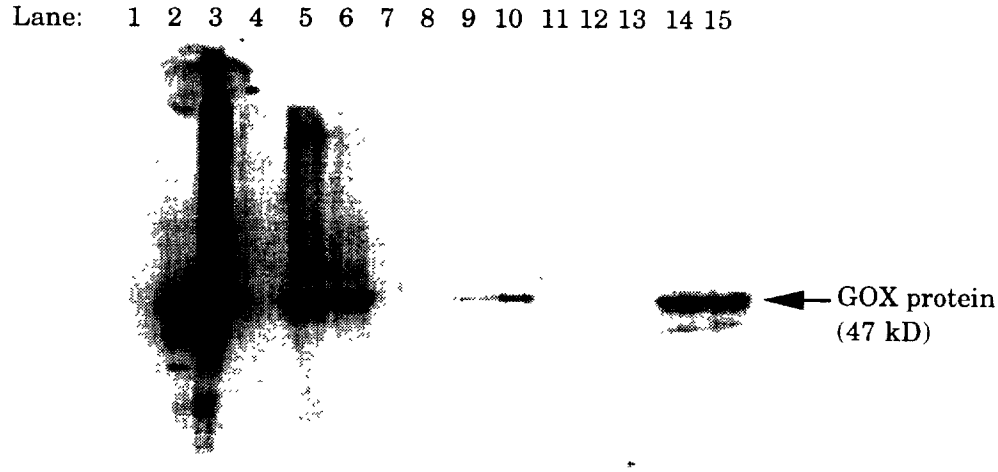
Figure 3. Western blot analysis of CP4 EPSPS protein in corn tissue extracts

Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

CP4 EPSPS protein
(47.6 kD)

Lane	Description
1	Sigma Color Molecular Weight Marker
2	≈20 ng of *E. coli*- produced CP4 EPSPS spiked into MON 819 leaf protein extract
3	≈20 ng of *E. coli*- produced CP4 EPSPS spiked into MON 818 leaf protein extract
4	Blank
5	MON 819 leaf protein extract
6	MON 818 leaf protein extract
7	MON 814 leaf protein extract
8	MON 813 leaf protein extract
9	MON 810 leaf protein extract
10	MON 809 leaf protein extract
11	MON 805 leaf protein extract
12	MON 802 leaf protein extract
13	MON 801 leaf protein extract
14	≈20 ng of *E. coli*-produced CP4 EPSPS
15	Sigma Color Molecular Weight Marker

Figure 4. Western blot analysis of GOX protein in corn tissue extracts.



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

Appendix II

CONFIDENTIAL BUSINESS INFORMATION

DNA Sequence of Plasmid PV-ZMBK07

Pages A00109 to A00117 contain Company Confidential material and may be found at the end of this submission

Appendix III

Assessment of the Likelihood of Lepidopteran Resistance with Insect-Protected Maize and Management Strategies

Introduction

Monsanto has developed an environmentally compatible alternative for control of lepidopteran insect pests by genetically modifying maize plants to produce the insecticidal protein CryIA(b) from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*). The CryIA(b) protein encoded by the vector used to produce the Insect-Protected maize varieties is identical to that found in nature and in a number of commercial *B.t.k.* formulations registered for almost 30 years in Europe and over 30 years by the United States Environmental Protection Agency (EPA). Field experiments conducted in the U.S. corn belt since 1992 and in Europe (France and Italy) since 1994 have demonstrated that Insect-Protected maize plants guard against foliage feeding and stalk tunneling from the European corn borer (ECB) [*Ostrinia nubilalis* (Hübner)]. ECB is the major lepidopteran pest for maize and causes average losses of five to seven percent of the harvested production in Europe and the U.S.A. (Gay, 1993).

In nine field experiments conducted by university cooperators during 1994 in the U.S. and in two yield comparison field experiments in France in 1995, Insect-Protected maize plants were essentially undamaged at all sites by native and manually infested ECB populations. The prevention of damage resulted in significantly more yield at harvest than non B.t.k.-protected maize in Nebraska (14-30%), Kansas (12-17%), Iowa (17%), Missouri (12-15%), Illinois (9%) and in France (15-40%) The elimination of significant ECB injury and consequent yield protection demonstrated with Insect-Protected maize, upon commercial introduction will lead to a reduction in the farmers dependence on conventional chemical insecticides for ECB control. Currently, growers in France and the U.S.A. treat approximately 14% and 10.4%, respectively, of the total maize acreage to combat ECB damage. Chemical insecticide usage is limited by the costs associated with treatment (product, application, and scouting), the relative risk of ineffectiveness of chemical insecticide-based management systems, or the impracticability of the treatment (Italy). Elsewhere, significant yield reductions are frequently caused by ECB but these yield losses cannot be economically recovered using traditional ECB management practices. Insect-Protected maize provides superior control of ECB and will provide important benefits to growers, society, and the environment. Insect-Protected maize will reduce application of chemical insecticides for targeted pests and will reduce the farmer's

time and effort spent on insect control. Reduced pesticide use also will decrease farmer exposure to chemical insecticides and decrease the environmental impact of insecticides on the agro-ecosystem.

Chemical insecticides like pyrethroids are relatively non-specific and decrease the diversity of the ecosystem by killing beneficial predatory and parasitic insects (Roush & Tingey 1993, Van den Bosch & Stern 1962). Because the CryIA(b) protein produced by Insect-Protected maize plants is not active against these beneficial insects, beneficial populations are expected to rise significantly in fields planted with genetically improved plants. For instance, the number of beneficial insects were shown to rise significantly in fields of insect-protected Newleaf™ potatoes and cotton expressing the Bollgard™ gene, compared to conventional potatoes and cotton not expressing a *B.t.k.* protein (Reed *et al.* 1992). Preserving the beneficial population and agro-ecosystem diversity enhanced the biological control of both target pests and non-target pests such as mites, aphids, and leafhoppers in insect-protected Bollgard cotton, Newleaf potatoes, and recently in Insect-Protected maize (Bowling *et al.* 1994).

The benefits of insect-protected maize have generated considerable interest among entomologists who are justifiably concerned about the potential for insect resistance to the *B.t.k.* protein. Although insect resistance is not an issue particular to Insect-Protected maize, given the history of insect resistance to chemical insecticides and the evolution of resistance by some field populations of the diamondback moth in response to Bt microbial sprays on broccoli crops, Monsanto is committed to developing and implementing strategies to maximize the durability of the *B.t.k.* protein and Insect-Protected maize.

Decades of experience have taught entomologists that insect populations adapt, sometimes quickly, to even the best insecticides if those insecticides are not managed correctly. With the development of insect-protected Bollgard cotton and Newleaf potatoes, Monsanto has been involved for several years in research work on insect resistance management. In 1992, Monsanto established an expert advisory panel composed of leading pest and resistance management researchers from academia, USDA-ARS, and university extension to help develop effective insect resistance management strategies for Insect-Protected maize. This group (Table 1) has grown to include twenty-one individuals that have met at least three times annually to discuss issues related to Insect-Protected maize performance and strategies for managing insect resistance. Monsanto's strategies discussed in this document are the result of extensive consultations with these recognized experts in insecticide research and development, maize insect biology, and insect resistance management. Moreover, field experiments, laboratory research, and computer simulations are on-going to evaluate the most effective, practical, and sustainable strategies for guarding against insect resistance to the *B.t.k.* protein.

In France, research is currently on-going on native insect population sensitivity to the *B.t.k.* protein and laboratory tests are in progress at the Institut National de la Recherche Agronomique (INRA) to determine resistance sensitivity of these populations. Although Monsanto has not been involved at the time these studies

were initiated, Monsanto is willing to collaborate with other academic and industry laboratories. This takes place in Monsanto's precautionary approach, described in this document, for the management of the risk of insect resistance.

The U.S. Environmental Protection Agency's Office of Pesticide Programs established the Pesticide Resistance Management Workgroup (PRMW) in August of 1992 to review plans for insect resistance management submitted by registrants in support of new conventional, biological, or genetically engineered plant pesticides (Matten & Lewis 1995). The PRMW identified seven elements which compose an adequate insect resistance management (IRM) plan. These components were subsequently approved on March 1, 1995 by a subpanel of the FIFRA Scientific Advisory Panel (Matten & Lewis 1995). These seven elements include:

- A - Knowledge of pest biology and ecology
- B - Gene deployment strategy; e.g.: full-season, constitutive, optimal dose *B.t.k.* expression to control insects heterozygous for resistance alleles
- C - Refuges to support the development of *B.t.k.* susceptible insects
- D - Monitoring and reporting of incidents of pesticide resistance development
- E - Employment of integrated pest management practices that encourage ecosystem diversity and provide multiple tactics for insect control
- F - Communication and education plan
- G - Development and deployment of products with alternative modes of action

The purpose of this report is to describe the IRM plan to be implemented both in Europe and the U.S. coincident with the introduction of Insect-Protected maize by Monsanto and seed company licensees. In addition to the elements listed above, the following is also a component of our comprehensive IRM programme for Insect-Protected maize:

- H - Actions to be taken if localised insect resistance occurs after commercial introduction of Insect-Protected maize.

Insect Resistance Management Plan for Insect-Protected Maize

● A - *Knowledge of Pest Biology and Ecology*

In order to implement pest management approaches, it is important to understand target pest biology, population ecology, mating behaviour, reproductive strategies, gene flow, and intercrop movement of polyphagous insects. To date, we have focused on the European corn borer as the primary target.

In the west of France, northern Italy and over most of the corn belt in the U.S., ECB typically completes two generations each year, but in warm years may complete

a partial to full third generation. In the east of France and northern maize growing areas in the U.S. and higher elevations, ECB may only complete one generation each year, whereas in southern U.S. areas ECB usually completes more than three generations annually.

a) Movement from plant to plant and implication for resistance management

After emergence, ECB neonates move from plant to plant, either between maize plants or between maize plants and weeds (Ross & Ostlie 1990, Hellmich personal communication). In the context of insect-protected maize, such a behaviour also increases the probability that larvae will encounter feeding sites more suitable for survival, such as plant tissues with intermediate or decaying *B.t.k.* protein content (if present) or weeds. In either case, ECB with intermediate levels of *B.t.k.* resistance would have an advantage compared to susceptible larvae feeding only on Insect-Protected maize with optimal dose expression. Interplant larval movement has been studied more exhaustively in conjunction with the potential deployment of random mixtures of *B.t.k.* expressing and non-expressing plants (Davis & Roush 1995, Mallot & Porter 1992). Davis, Roush, and Gould (personal communication) have concluded that interplant movement of ECB larvae is significant and may increase rather than delay resistance development in seed mixtures or in plants with incomplete or decaying *B.t.k.* protein expression.

b) adults dispersal and implication for resistance management

After emergence of the first generation in the spring or subsequent generations during the summer months, ECB adults disperse and congregate in action sites (typically mixtures of grass and broadleaf weeds) to avoid the hot, desiccating climatic conditions and to mate (Showers *et al.* 1976). ECB adults are known to disperse at least 800 meters (Showers 1993) and bivoltine populations may disperse up to 32 kilometres in a single year (Showers *et al.* 1995). This dispersal behaviour of ECB will help to counter any increase in local resistance gene frequency resulting from selection within an insect-protected maize field by favouring the reintroduction of susceptible alleles from nearby conventional maize. Maintaining susceptible alleles in the insect population is critically important for avoiding insect resistance development.

c) Plant hosts

ECB are polyphagous and are known to host numerous crop and weed species. This behaviour helps to decrease the likelihood of resistance development in regions where multiple host plants are available. The occurrence of host plants without *B.t.k.* expression will provide an escape from the *B.t.k.* protein for some individuals in the population and reduce the overall selection intensity for *B.t.k.* resistance. Hellmich and Showers (1994) evaluated the fecundity, survival, and development of ECB in weed patches (action sites). They demonstrated that common maize weeds such as giant and common ragweeds (*Ambrosia artemisifolia*), velvetleaf (*Abutilon teophrasti*), pigweed spp. (*Amaranthus* sp.), smartweed spp. (*Chenopodium* sp.), giant foxtail (*Setaria*), and fall panicum (*Panicum*) supported ECB population development and may contribute to a significant non-maize refuge.

● B - *Gene Deployment Strategy: Full-Season, Constitutive, Optimal Dose B.T.K. Expression*

By definition, the optimal-dose approach asserts that resistance can be delayed by delivering a dose sufficiently high to kill all or nearly all of the resistant heterozygotes, the most common carriers of resistance genes, and perhaps many of the resistant homozygotes. Therefore, because the susceptible homozygotes and most, if not all, of the resistant heterozygotes die, resistance is functionally recessive (Roush & McKenzie 1987, Roush & Daly 1990). This approach is particularly successful when the frequency of resistance alleles is low, less than 10^{-3}, and a significant fraction of the population escapes exposure altogether. At this resistance gene frequency, resistant homozygotes are rare (1 in 10^6 individuals). Rare resistant individuals are more likely to mate with susceptible individuals escaping exposure, i.e. in the refuge, than with other resistant individuals. As a result, resistance genes will thereby be maintained in heterozygous genotypes which, by definition, are susceptible to the optimal-dose (Tabashnik & Croft 1982, Roush 1989, 1994).

To be effective, plants expressing the *B.t.k.* protein must deliver a sufficiently high dose all season long in all important tissues, i.e. in all the tissues of the plant where the target insect may feed. Expression targeted at some but not all important tissues will permit some individuals in the population to survive. Because some insects such as ECB move significantly about the plant, incomplete plant expression increases the risk of resistance by selecting for more resistant genotypes.

Similarly, if expression decreases significantly over the course of the growing season, *B.t.k.* protein levels may decay to levels that discriminate between susceptible and partially resistant individuals. If this occurs, resistance could occur more quickly because resistant genotypes survive while susceptible genotypes die. Failure to provide season-long optimal dose expression of *B.t.k* protein in key maize tissues, such as the leaves, stalk, ear shank, husk, pollen, silks, and kernels, may allow survival of ECB with intermediate levels of *B.t.k.* resistance (individuals assumed to be heterozygous for resistance alleles) in weakly expressing tissues and lead to resistance development. The phenomenon of pesticide decay following conventional insecticide application is well established and is a recognized contributing factor to the development of insect resistance. Deployment of genetically engineered plants with incomplete *B.t.k.* expression or plants with decaying *B.t.k.* protein could mimic conventional insecticide management systems and promote rather than delay development of insect resistance.

Demonstration of the optimal-dose is impossible unless resistant insects exist to test; even then, there may be an alternate resistance mechanism not yet discovered that may resist even the highest expressing *B.t.k.* plants. Instead, the real goal is to express the *B.t.k.* protein at consistently high levels throughout the growing season and in all important tissues, such that all insects exposed are killed. Statistically, if survivors are routinely found on insect-protected plants, it is almost certain that the plants are not providing a consistently high dose.

Monsanto and its cooperators have demonstrated that the events (including MON 810) selected for development of commercial Insect-Protected maize hybrids produce the CryIA(b) protein at levels sufficient to provide season-long control of ECB. CryIA(b) protein is produced at sufficiently high levels in all important tissues: foliage, leaf sheath, stalk, ear shoot, ear shank, pollen, silks, and kernels. These results are consistent with an optimal-dose approach. To effectively reduce the risk of resistance development, it is also necessary that a significant fraction of the insect population remain unexposed to the CryIA(b) protein i.e. that a refuge for susceptible insect is provided. The optimal-dose approach coupled with a significant refuge is supported by many experts because this approach avoids the problems associated with pesticide decay and imperfect plant coverage associated with topically applied chemical and microbial insecticides.

In some cases, expression of a low-moderate dose can cause minor reductions in pest populations and, for bi-voltine or multi-voltine pests, effectively reduce the number of generations per year (McGaughey & Whalon 1992) by slowing larval development. This option may be attractive for sporadic and secondary pests, whose populations are usually below the economic threshold or are held in check by other control agents, such as biological agents. In such cases selection for resistance is low. However, for persistent pests like ECB, the low-moderate dose approach may be unappealing since large populations and chronic damage are evident to the farmer most years. Because Insect-Protected maize is targeted at ECB, current expert opinion favours a full season, constitutive, optimal dose approach, coupled with an adequate refuge, for managing ECB resistance with insect-protected maize (Gould 1994, Roush 1989).

At present, no one has identified a single gene or multiple genes which could confer high level insect resistance to the *B.t.k.* protein produced by Insect-Protected maize. Therefore the frequency and dose sensitivity of insect genes that confer resistance is unknown. Since optimal-dose is more an objective than a fixed-target, we believe that the best approach is to pursue a product with the highest dose possible

Further Research

Environment also is known to effect plant gene expression. In 1995, Monsanto personnel and academic cooperators collected samples from key maize tissues during the course of the growing season. The objective of this study is to correlate outstanding field efficacy with full-season, constitutive, *cryIA(b)* gene expression, in all major Insect-Protected maize tissues. The samples have been analysed by a quantitative enzyme-linked immuno sorbance assay (ELISA) and are presently being validated by insect bioassay. Variation in *cryIA(b)* gene expression due to differences in environment (Genotype x Environment effects) will also be evaluated.

● C - *Refuges To Support Population Of B.t.k. Susceptible Insects*

The refuge is a necessary and integral component of the optimal dose IRM strategy (Gould 1986, Roush 1989). For any product expected to kill all susceptible individuals and most if not all of the resistance heterozygotes in an insect population (optimal-dose), the purpose of a refuge is to replenish the susceptible alleles removed by selection. This implies the absence of insect control by any insecticide effective against the target pest in the refuge area. In a sense, the susceptible alleles provided by the refuge "dilute" any resistance alleles that remain after selection, making them rare again (Tabashnik 1994, Gould 1994, Roush 1994). With Insect-Protected maize, the presence of a large number of susceptible individuals arising from a refuge increases the likelihood that a rare resistant homozygote (RR) will mate with a susceptible homozygote (SS). Since the progeny (RS) of this mating are all susceptible (definition of optimal-dose), resistance is held in check.

Since it is clear that refuges are a critical component of IRM, at issue is how to ensure that an appropriate and significant refuge exists for insect populations exposed to Insect-Protected maize. Several potential refuges have been described for ECB:

Natural Refuges: Weeds and other host crops
ECB can survive on over 200 species of plants (Hodgson 1928). Hellmich and Showers (1994) surveyed common maize weeds for ECB egg masses and larvae and found both prevalent on giant and common ragweed (*Ambrosia artemisifolia*), velvetleaf (*Abutilon teophrasti*), pigweed spp. (*Amaranthus* sp.), smartweed spp. (*Chenopodium* sp.), giant foxtail (*Setaria*), and fall panicum (*Panicum*). During the fall, they confirmed the presence of fifth instars on giant foxtail, fall panicum, Pennsylvania smartweed, and barnyardgrass (*Echinochloa cruss-galli*). They found the mixed-weed environment provided excellent shelter, and as one species senesced, the larvae could move within these weed patches to other hosts. Other hosts include: millet, sorghum, cotton, and tobacco.

Unmanaged Refuges: Neighbouring maize fields without the *B.t.k.* gene
Most experts agree that the most important refuge for insect-protected maize is non *B.t.k.*-protected maize. Farmers will continue to plant non *B.t.k.*-protected maize, at least until Insect-Protected maize is more widely available. On a regional scale, it is unlikely that Insect-Protected maize will dominate the market. It is expected that price vs benefit, brandname competition, and the availability of more elite, higher yielding hybrids without the *B.t.k.* gene, will continue to provide barriers to full market penetration, at least for the next 5-10 years. On a local scale, however, there is a potential for localised areas with a consistent ECB threat to more rapidly adopt Insect-Protected maize. In these areas, it is imperative that a refuge be provided.

Managed Refuges: On-farm maize fields or other host crops without the *B.t.k.* gene
Deliberate planting of non *B.t.k.*-protected maize or other agronomic crops that are suitable ECB hosts will ensure that a refuge exists to support development of susceptible insects; and ensure that any rare resistant individuals that may arise

from Insect-Protected maize fields are more likely to mate with susceptible individuals arising from the refuge. In areas where ECB consistently reduce maize yield, this may be the best approach for ensuring a refuge. There are several managed refuge options:

Adjacent fields:	non *B.t.k.*-protected maize or other suitable alternate crops grown in adjacent fields or in close proximity.
Refuge strips:	non *B.t.k.*-protected maize planted in adjacent rows within the field or at the field edge.
Seed mix:	non *B.t.k.*-protected maize seed may be premixed with Insect-Protected maize at a predetermined ratio.

Natural, unmanaged, and managed refuges all are important components of an IRM programme. Given these refuge options, more research is needed to determine the size of the refuge needed and how to spatially structure the refuge.

One approach may involve alternate planting of non *B.t.k.*-protected maize with several rows of Insect-Protected maize. At the far end of the spectrum, this would be equivalent to planting adjacent fields of Insect-Protected and non *B.t.k.*-protected maize. A potential short-coming of this approach is that the non *B.t.k.*-protected refuge fields may sustain significant yield losses (Alstad & Andow 1995), especially in areas of high ECB population density.

Recent modelling work by Alstad and Andow (1995) suggests that a biased patchwork planting arrangement may significantly delay the onset of ECB resistance development with Insect-Protected maize and at the same time reduce damage in non *B.t.k.*-protected maize refuges. This model is based on the fact the ECB females preferentially oviposit on taller plants. Since farmers typically plant full-season and shorter maturity hybrids to spread environmental risk and harvest dates, planting faster growing, shorter maturity Insect-Protected hybrids could preferentially attract ECB females. The oviposition preference for Insect-Protected maize is expected to cause disproportionate mortality in the local ECB population resulting in significant protection of the refuge. Although this model poses some interesting possibilities, it remains to be validated, and planting non *B.t.k.*-protected maize as the major full-season hybrid may not fit well with current agricultural practices.

Seed mixes of Insect-Protected and non *B.t.k.*-protected maize are often identified as a potentially straight-forward effective method for establishing a managed refuge. Given the importance of a local refuge when planting Insect-Protected maize, our initial approach was to investigate seed mixes. Seed mixes would provide a means of easily managing and enforcing the resistance management programme by marketing Insect-Protected maize only as a mixture. Because interplant movement exceeding 20 percent (i.e. 20% of the larvae feed on more than one plant) can significantly decrease the value of the seed-mix approach (Mallet & Porter 1992, Tabashnik 1993, Roush 1994), Monsanto initiated cooperative research with Cornell University (P. Davis and R. Roush) and North Carolina State University (F. Gould)

to investigate the effectiveness of mixtures for IRM in maize. These researchers studied ECB dispersal and survival over time in mixtures of different ratios of Insect-Protected and non *B.t.k.*-protected maize. Based on two years of research, both groups have concluded that interplant movement of ECB larvae is too great and may increase rather than delay resistance development (Davis & Roush 1994 1995, Gould 1994).

Because the occurrence of a local refuge is an integral component of the optimal-dose gene deployment strategy, and natural or unmanaged refuges may be inadequate in areas of frequent and significant ECB densities, Monsanto recommends a managed refuge approach. To ensure that there are acceptable numbers of susceptible ECB adults emerging within the local refuges, Monsanto will implement a market approach that encourages farmers to continue to plant a portion of their land with non *B.t.k.*-protected hybrids. The objective of this approach will be to make certain that an non *B.t.k.*-protected refuge area is implemented by the farmer. Monsanto will work closely with seed company licensees to establish and implement this approach.

Further Research

The refuge is a key component of the optimal-dose strategy, yet the contribution of natural and unmanaged refuges, the size of the refuge needed, and recommendations for deployment of managed refuges need to be further investigated. Monsanto is cooperating with Universities and Research Institutes, to quantify the contribution of susceptible ECB from natural and managed refuges. Our ultimate goal is to provide farmers with practical recommendations for refuge design and implementation

The likelihood of Insect resistance development will be influenced strongly by mating behaviour and dispersal within and between fields of Insect-Protected and non *B.t.k.*-protected maize. Little is known about ECB dispersal especially over relatively short distances. Showers (1993) data suggest ECB dispersal of at least 800 meters, but dispersal is confounded by maize phenology and the relative attractiveness of different maize stages. Monsanto will continue to support academic research directed at understanding ECB dispersal, mating behaviour, and population genetics parameters such as the neighbourhood effective population size (N_e), mean displacement (S), and population subdivision (F_{ST}). This information will improve the utility of ECB computer models for predicting the likelihood of ECB resistance to the *B.t.k.* protein given different IRM strategies.

● D - *Monitoring And Reporting Of Incidents Of Pesticide Resistance Development*

IRM is essentially the management of a finite natural genetic resource — susceptible genes or alleles in the population — and is analogous to management of other natural resources such as timber and water (McGaughey & Whalon 1992).

Successful management of this resource requires early detection of resistance individuals (if they develop), timely and accurate estimates of population shifts toward resistance development, and initiation of rapid containment policies in the event of localized product failure (McGaughey & Whalon 1992, Georghiou & Mellon 1983). Two monitoring approaches have received considerable attention:
(1) **surveillance**: directed at insects within fields of Insect-Protected maize; and
(2) **monitoring programmes**: directed at ECB populations.

Surveillance

Surveillance must be the responsibility of all agriculturalists, because of its importance for detection of the early stages of insect resistance. Early detection of resistant individuals is important for successful resistance containment since it allows targeting of the most intensive and effective monitoring and containment policies where they will have the greatest chance of success (Georghiou & Mellon 1983). The first line of surveillance is necessarily the farmer, since he will likely be the first to identify resistant individuals, alarmed by decreased product performance or failure in the field.

Monitoring Programmes

Monitoring programmes can have two different purposes (D. Alstad, personal communication).

a) First, monitoring programmes can offer a mean of predicting the potential for resistance development. In principle, accurate local estimates of evolutionary potential would allow implementation of management tactics to delay or prevent further resistance development. Estimation of evolutionary potential relies on either phenotypic or genetic assays of field collected individuals from geographically representative areas.

- Phenotypic assays use dose-response analyses to compare LC_{50} values of putative resistant individuals to baseline data from susceptible populations. The effectiveness and accuracy of this approach is very much contingent upon establishment of complete dose-response analysis of insect populations from geographically diverse areas. If possible, baseline estimates of *B.t.k.* protein susceptibility need to be determined prior to deployment of the protein. This is not strictly possible with Insect-Protected maize, since farmers have used Bt products, in varying degrees, for insect control for decades.

The assessment of the susceptibility to the *B.t.k.* protein of existing ECB populations in France is part of a study managed by the I.N.R.A. laboratory of Versailles and Monsanto proposes to collaborate to this study. To develop baseline data for ECB susceptibility to *B.t.k.* insecticidal proteins across diverse geographical areas in the U.S., Siegfried *et al.* (1993, 1994) surveyed nine Nebraska ECB populations for susceptibility to the *B.t.k.* protein. The populations were evaluated for susceptibility to the CryIA(b) protein, the same insecticidal protein produced by Insect-Protected maize. The LC_{50} values ranged 6-fold, from 0.73 $\mu g\ cm^{-3}$ diet to 4.22 $\mu g\ cm^{-3}$ diet. The slopes of the dose/mortality curves were

similar and reflect the genetic heterogeneity of the populations typical of the response of most insects to microbial insecticides. The range in susceptibility among the geographically distinct populations is similar to the range reported for other insects evaluated with microbial proteins. For example, with the CryIA(c) protein, the LC_{50} values for the tobacco budworm ranged 8-fold, from 0.04 $\mu g\ ml^{-1}$ diet to 0.32 $\mu g\ ml^{-1}$ diet; and the LC_{50} values for the corn earworm ranged 8-fold, from 0.45 $\mu g\ ml^{-1}$ diet to 7.39 $\mu g\ ml^{-1}$ diet (Stone & Sims 1993). In a similar study, Everich et al. (1994) identified a 5.2-fold range in susceptibility of Colorado potato beetle populations to the *B.t.* subsp. *tenebrionis* protein. Thus, the 6-fold range in susceptibility exhibited by the ECB populations does not demonstrate that resistance to the *B.t.k.* protein has developed but does imply that genetic diversity exists. Accordingly, it is important that resistance monitoring be started with the commercial introduction of Insect-Protected maize to detect any changes in the susceptibility of target insect populations to the *B.t.k.* protein. Furthermore, since a range in susceptibility to *B.t.k.* proteins exists naturally, population comparisons cannot be used to determine shifts in susceptibility. Shifts must be monitored over time in each geographically distinct population.

- Genetic assays rely on identification of some genetic trait or marker linked to resistance. Thus, resistance must have already occurred to make use of this approach. In principle, laboratory selection for resistance could result in identification of a marker to track that resistance, but considering the multitude of different resistance mechanisms possible, reliance on monitoring for any one trait is of questionable usefulness.

There are two major short-comings associated with using monitoring as a predictive tool. First, LC_{50} estimates of insect sensitivity may range 5 to 20-fold due to the natural diversity in insect populations. Therefore, LC_{50} estimates are likely to be so insensitive that resistance alleles will be quite common by the time phenotypic effects are observed through monitoring. Second, genetic protocols that seek to identify and monitor resistance genes, perhaps developed from laboratory selection assays, also may be unacceptable since they may lead to a false sense of security if the wrong gene is monitored. The greatest utility of these approaches for preventing or containing resistance is when they are used in support of surveillance.

b) The second purpose of a monitoring programme is to detect resistance as it occurs in the field. Under this scenario, monitoring is analogous to surveillance. The major differences between the two are in levels of expectation and cost. If the goal of the monitoring programme is detection of rare resistant individuals, then the magnitude of an organized monitoring programme would be staggering. The identification of rare resistant insects would require saturation collection of large numbers of insects from many sites and dose-response analysis. In addition, these insects would have to be laboratory reared under quarantine procedures to prevent latent *Nosema* infection. As pointed out by the insecticide research community, despite the considerable effort made to obtain and assay large numbers of insects, the monitoring-based assay is frequently ambiguous (Miyata 1983).

In summary, the expectation of finding a rare resistant individual via monitoring is low, while the cost may be extremely high. The recommended strategy is to rely on surveillance to detect any putative resistance and, if this occurs, focus monitoring and implement efforts in that area to contain the resistance.

Diagnostic Tools for monitoring

For chemical insecticides, the discriminating dose assay was developed to reduce the cost and labour associated with surveillance and monitoring of insect resistance (Georghiou & Mellon 1983). The discriminating dose assay utilises a single discriminating dose to separate resistant individuals from susceptible individuals. The goal is to determine a single protein dose that kills all susceptible individuals but allows survival of individuals that exceed some threshold level, i.e., the upper 95% confidence limit of the LD_{99}. For classical insecticides that kill on contact, development of the discriminating dose assay is attractive since individual insects can be evaluated for resistance in the field. The insect can be added to an insecticide-coated vial and scored for survival after only a few minutes. Because *B.t.k.* insecticidal proteins require ingestion and several hours or days for mortality, development of a discriminating dose assay for Insect-Protected maize will require identification of a suitable test substance (Bt protein or plant tissue) and appropriate measurement criteria (mortality or growth inhibition). In addition, because ECB larvae become more tolerant to the *B.t.k.* insecticidal protein as they mature, it is likely that only assays based on neonates will provide consistent, reproducible results. If so, the assay procedure would require field collection and laboratory rearing to make a reliable determination of putative resistance.

Conclusion

By the time of Insect-Protected maize commercial introduction, Monsanto and the seed companies licensees will implement (via seed purchase recording system) a focused surveillance programme designed to detect any indications of insect resistance, confirm resistance, and implement a monitoring programme to assess for any increased frequency or level of resistance. Quick assessment would allow any insect resistance suspicion to be confirmed or explained, and if verified, tactics could be immediately implemented to minimize the likelihood that resistance spread to nearby fields (see paragraph H-). Moreover, if insect resistance is identified, modifications of existing IRM strategies may be necessary.

Further Research

Monsanto proposes to participate to ECB baseline susceptibility studies in Europe and will continue to support the ECB baseline susceptibility studies by Dr. Blair Siegfried at the University of Nebraska. The 1993/94 data are sufficient to establish a baseline *B.t.k.* protein susceptibility for ECB in Nebraska. In 1995, ECB collections were expanded to Iowa, Illinois, Indiana, Pennsylvania, North Dakota, North Carolina, and Italy; these analyses are in progress. Preliminary results demonstrate that the ECB baseline susceptibilities measured at these sites, including Italy, are similar to the established values from Nebraska.

Monsanto will continue the development of a discriminating dose assay. We will apply our experience gained in development of similar assays for monitoring of tobacco budworm [*Heliothis virescens* (Favbricius)] sensitivity to the *B.t.k.* protein in cotton expressing the Bollgard gene.

Monsanto is willing to collaborate with other industry and academic laboratories to develop sampling methodologies, surveillance and monitoring approaches for effective and economical detection of ECB resistance development.

Monsanto will develop educational programmes and communicate to farmers the importance of surveillance and early detection of resistant insects. We will develop a surveillance and monitoring programme based on the early years of experiences, results of research with academic collaborators, and the advice of government and industry experts. Monsanto is intending to participate in the surveillance and monitoring framework necessary to reasonably assure detection of true resistance development.

● E - *Employment Of Integrated Pest Management Practices That Encourage Ecosystem Diversity*

Insect-Protected maize is not a stand-alone measure for insect problems in maize. Decades of experience have taught entomologists that insect populations adapt, sometimes quickly, to even the best insecticides if those insecticides are not managed correctly. Integrated pest management (IPM) was developed in large part as a result of industry experiences with chemical insecticides. Monsanto is committed to maintaining the value of Insect-Protected maize and intends to learn from the experiences of the past, to minimise the likelihood of insect resistance development. We believe that the same IPM principles and practices developed for insecticides may be applied to Insect-Protected maize.

IPM may be defined as the utilisation of different control strategies that maintain insect populations below economic thresholds without eliminating them (Ebora & Sticklen 1994). Pertinent components of IPM include: host plant resistance, biological control, chemical control, and agronomic practices (Wiseman 1994, Luckmann & Metcalf 1982).

Host plant resistance (HPR) and biological control are viewed by many as the corner stones of an IPM programme. HPR insures that the first line of defense, the plant, is protected from insects. Biological control seeks to keep the pest in check through the use of naturally occurring disease organisms and beneficial insects such as parasitoids and predators. The adoption of Insect-Protected maize will result in an increased abundance of beneficial insects in the agroecosystem due to a reduction in the use of insecticides for ECB control and an emphasis on the first line of pest defense: the plant.

The sometimes detrimental effects of chemical insecticides on beneficial insects is well documented. Natural enemies aid in the control of target and non-target pests and help to effectively maintain pest populations in check. Use of broad spectrum chemical insecticides, even when part of a planned IPM system, reduce beneficial predatory and parasitic insect densities to very low levels, effectively rendering these beneficial insects useless for controlling crop damaging pests. For instance, in some areas of Europe and of the U.S. corn belt, spider mites are an important secondary pest of maize. Chemical insecticide applications to reduce ECB damage to maize often result in destruction of the beneficial insects that normally prevent secondary outbreaks of the spider mite. As a result, spider mite populations often increase unhindered, causing significant maize damage. When Insect-Protected maize is used for control of ECB, the natural enemies of spider mites and other pests are preserved, making it possible for these pests to be managed naturally, as part of an IPM-based management programme.

Local beneficial insect populations also provide the first line of defense against potential *B.t.k*-resistant pests. In the event that an ECB larvae is genetically resistant to the *B.t.k.* protein, the presence of various natural enemies of ECB will help to remove the resistant individual and its resistance gene(s) from the population. Furthermore, due to the mobility of most beneficial insects, their preservation in maize can have positive effects on pests in neighbouring fields of maize or other crops.

Of all pest control measures, chemical controls have provided growers and consumers with some of the greatest successes and biggest disappointments. This is largely due to an over reliance of the grower on a single, all-encompassing, control measure. Chemical control will continue to play a role in the cropping system to control other non-target pests, but it will be used more strategically since current Integrated Pest Management practices seek to de-emphasize chemical control and re-emphasize Host Plant Resistance and biological control.

Agronomic practices also will remain an important component of an IPM-based IRM strategy. The major agronomic practice relevant to ECB resistance management is planting strategy. The farmer will decide which hybrids to plant, whether they are Insect-Protected or non *B.t.k.*-protected, and will be advised to implement the most effective refuge option. Other agronomic practices such as destruction of the overwintering habitat of ECB and other insects by shredding of the stalks and ploughing also will continue to be important.

Conclusion

The use of Insect-Protected maize is consistent with recognized IPM practices and fits logically within the framework of HPR (Ebora & Sticklen 1994). The only difference between classical HPR and Insect-Protected maize *B.t.k.*-based HPR is the addition of the *B.t.k.* gene Accordingly, utilizing Insect-Protected maize as an integral component of IPM has a major advantage compared to chemical insecticides; the *B.t.k.* protein is readily biodegradable and environmentally friendly.

Furthermore, since the *B.t.k.* protein is produced in plant tissues that are fed on by the target insect, the result is a more effective, directed delivery of the insecticidal protein to the pest. Finally, the *B.t.k.* protein is very species specific and produces no negative effects on non-target beneficial insects. Many have argued that the plant should be at the centre of an IPM-based programme (Ebora & Sticklen 1994, Wiseman 1994, Hoy 1993). Insect-Protected maize, along with future generations of genetically improved crops, are a major step toward attainment of that goal.

Further Research

Studies were conducted by Monsanto its cooperators from 1993 to 1995 to determine the effects of pest control practices, including Insect-Protected maize, on arthropod population dynamics. Data were collected from sites in Nebraska, Iowa, Missouri, and Kentucky, in the U.S.; and in southern France (see Appendix IV). The results demonstrate that Insect-Protected maize is harmless to all beneficial insects studied: *Orius*, nabids, big-eyed bug (*Geocorius*), lady beetles (*Hippodamia* and *Coleomegilla*), *Hippodamia*, and spiders. Furthermore, spider mite densities increased above the economic threshold in maize plots receiving chemical insecticide applications and rescue treatments with a miticide were necessary to slow the progression of the mites. Spider mites remained at low densities in the Insect-Protected maize plots, presumably as a result of beneficial insects inhabiting the Insect-Protected maize that were absent from the non *B.t.k.*-protected maize treated with chemical insecticides.

Monsanto will continue to participate in research that will help to integrate Insect-Protected maize into an IPM-based maize pest management programme. We also will develop guidelines for different cropping systems such as conventional tillage and conservation tillage. We expect that economic injury levels for non *B.t.k.*-protected maize in refuges and cultural control methods such as ploughing and shredding may change under different cropping systems.

● F - *Communication and Education Plan*

Maintaining the effectiveness of Insect-Protected maize for many years to come will depend on good product stewardship, grower awareness of the potential for insect resistance, regular and consistent communication at all levels of the product chain, and grower implementation and support of IRM recommendations. To be effective, farmers must understand more than the societal and environmental benefits of preserving the effectiveness of Insect-Protected maize (Kennedy & Whalon 1994). They must be convinced that the short and long-term value of Insect-Protected maize on their farm is their responsibility. Moreover, farmers must agree to manage insect resistance on their own farms.

Product Stewardship

Monsanto has invested considerable financial resources to discover and develop a novel approach for eliminating yield losses caused by the European corn borer and

other lepidopteran pests. To protect this investment and to insure long-term profitability, Monsanto also has invested heavily in the research and development of workable Insect Resistance Management strategies that may be implemented easily by the farmer. Insect-Protected maize provides an easy-to-use, economical, and safe alternative to chemical insecticides. We will provide support to farmers, consultants, and the seed industry to insure customer satisfaction for years to come.

Awareness of the Potential for Insect Resistance

Monsanto will develop educational tools that explain to growers how insect resistance develops. We also will work closely with crop protection agents, farmer-dealers, seed distributors, seed companies, universities, consultant organizations, grower organizations, and industry organizations, to develop training materials and literature that clearly explain the potential for insect resistance development. The growers will learn that insect resistance is a real possibility and should be taken very seriously.

Regular and Consistent Communication

Regular and consistent communication at all levels of the product chain is absolutely critical to gain grower acceptance of recommended IRM measures. Monsanto and seed companies licensees will communicate a simple but consistent message to grower organisations, seed distributors, farmer-dealers, farmer advisors, and farmers. We will enlist grower and industry organisations to assist in distributing this information to their members. Finally, IRM recommendations will be emphasised when the farmer purchases seed.

Farmer Implementation and Support of IRM Recommendations

Managing insect resistance is not possible unless workable IRM tactics are identified and implemented by farmers. Monsanto will continue to research and develop the best approaches for managing insect resistance. We will continue cooperative research with academics and specialists to simplify and optimise deployment of Insect-Protected maize. Farmers will be informed of the latest IRM strategies. In turn, farmers must follow the IRM recommendations. To successfully combat insect resistance development, all levels of the product chain, from the seed companies to the farmers, need to work together.

● G - *Development And Deployment Of Products With Alternative Modes Of Action*

Multiple gene and alternate gene strategies show promise for substantially delaying resistance development. To be effective, there must be a refuge and heterozygotes for both insect genotypes must suffer high mortality when the gene products are used independently (Gould 1988a, 1988b) If the insect population becomes resistant to one gene product, then the other gene product can be deployed in such a way that delays resistance to both gene products. There are several approaches: (1) by introducing the second gene after the first fails; (2) by rotating

between genes every year; (3) by mixing seed with each gene in the same bag; or (4) by putting both genes in the same plant. Modelling data indicates that each approach has value compared to a single gene, but the greatest benefit occurs when both genes are in the same plant (Roush 1994). Pyramiding or stacking genes in this way can achieve an enormous delay of resistance (>1000-fold).

An alternative approach, but not mutually exclusive, is to improve plant resistance to insects using conventional breeding methodology. This approach has been used widely by public and private maize breeders for over 60 years to improve maize resistance to ECB (Guthrie et al. 1984, Hudon 1984). Most modern maize hybrids are significantly more tolerant or resistant to ECB injury than hybrids of 30 years ago. Resistance to ECB whorl feeding is associated with at least six genes (Scott et al. 1966) and resistance to ECB sheath feeding is associated with at least seven genes (Onukogu et al. 1978). In addition to these resistance factors, maize is naturally resistant to ECB injury during the early stages of development (up to 25-30 cm for inbreds and 40-45 cm for hybrids) due to the production of DIMBOA. The net result of 60 years of maize breeding is that modern hybrids contain significant levels of ECB resistance, but major yield reductions continue to occur when ECB density is moderate to high and/or the maize is drought-stressed. For the best possible ECB control and for IRM, seed companies should develop hybrids with both the *B.t.k.* gene and native ECB resistance genes.

Although Monsanto is actively screening for novel insect control proteins, the best alternate gene source currently is other *B.t.k.* proteins with different site of action. We have identified a second *B.t.k* gene as our best option and are actively developing Insect-Protected maize lines expressing two different *B.t.k.* proteins that act by independent mechanisms.

Further Research

We have developed two lead lines expressing the second *B.t.k.* gene and have demonstrated that the maize plants produce this *B.t.k.* protein at levels sufficient to control ECB in a greenhouse experiment. This is an important result since in order to be effective the second *B.t.k* protein expressed in plants should also provide optimal-dose control similar to the CryIA(b) protein. The results to date also indicate that combining the two different *B.t.k* genes in the same maize hybrid has the potential to delay resistance for many years. The existing lines expressing the second *B.t.k* gene and backups in development will be evaluated for genetic and agronomic acceptability. This evaluation is designed to assess gene stability, insect efficacy, gene expression in plant tissues and over the course of the season, interaction with other agronomic traits (e.g.: maturity), and yield. If the technical performance is satisfactory, hybrids with both *B.t.k.* genes may be commercialised before the full adoption of single gene hybrids.

Because of the agricultural and environmental importance of Insect-Protected maize and other Bt products, and Monsanto's financial investment in developing and delivering Insect-Protected maize, we will continue to pursue alternate genes and

deployment strategies to reduce the likelihood of insect resistance and extend the product life of Insect-Protected maize.

● H - *Actions To Be Taken If Localised Insect Resistance Occurs*

Currently, there are no means available to confirm or refute the presence of insect resistance in the field. The best indicator of ECB resistance to Insect-Protected maize will be "higher than expected levels of ECB damage". Should this occur, Monsanto and seed company licensees will immediately investigate farmer's claims of product reduced efficacy. We anticipate that many claims of non-performance may be explained by 1) migration of large ECB larvae (requiring higher plant tissue consumption) from weeds to the Insect-Protected maize plants located adjacent to large weeds or along field perimeters and 2) the presence of non *B.t.k.*-protected maize seed in the planter following previous planting operations.

If all other potential explanations for ECB injury to Insect-Protected maize have been considered and the affected plants have been confirmed to express the *B.t.k.* protein, then Monsanto will implement a quick, proactive approach to contain putative insect resistance, in cooperation with the local crop-protection authorities. Our response will include the following actions:

- Intensify field surveillance for Insect-Protected maize efficacy in and around the potential "resistance epicentre" to define the boundaries of the affected area. We will enlist support from neighbouring farmers, crop-protection authorities, field consultants, and industry cooperators, as needed.
- Investigate farmer practices, Insect-Protected maize usage, and adherence to recommended IRM tactics within the affected area.
- Recommend to farmers and crop protection or extension agents near the "resistance epicentre" alternate control measures to reduce and control the local "resistant" population. These recommendations may include the use of chemical insecticides to reduce survival of "resistant" insects. Factors such as crop growth stage, presence of biological control agents, and weather will be considered when making appropriate recommendations.
- Advise farmers to shred stalks following harvest and incorporate crop residue into the soil to reduce overwintering survival of ECB.

Once initial fact-gathering and containment are completed, Monsanto will work with crop protection authorities or university extension, government experts, to determine appropriate follow-up actions. These actions may include one or more of the following steps:

- Inform customers and extension agents within the affected area that ECB resistance development is suspected and inform them of the region impacted.

- Increase field surveillance in and around the "resistance epicentre", and collect representative insects for dose-response bioassay analysis.
- Recommend alternative measures to reduce or control ECB populations in the affected area in subsequent years.
- Assess the Insect-Protected maize usage and planting practices that may have increased the likelihood of insect resistance development.
- Recommend modification of existing refuge strategies based on the most current and accepted research findings.

Conclusion

Monsanto is committed to the implementation of insect resistance management strategies. This is likely to require a specific market approach for the Insect-Protected maize and Monsanto is currently working with seed companies licencees and distribution network to set-up the framework for implementation.

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Table 1. Monsanto's Expert Advisory Panel for managing insect resistance.

Dr. Randy Higgins	Kansas State University
Dr. Larry Buschman	Kansas State University
Dr. Jerry Wilde	Kansas State University
Dr. Dean Barry	USDA-ARS
Dr. John Foster	University of Nebraska
Dr. John Witkowski	University of Nebraska
Dr. Bob Wright	University of Nebraska
Dr. Blair Siegfried	University of Nebraska
Dr. Rick Hellmich	Iowa State University
Dr. Les Lewis	Iowa State University
Dr. Marlin Rice	Iowa State University
Dr. Kevin Steffey	University of Illinois
Dr. David Andow	University of Minnesota
Dr. Don Alstad	University of Minnesota
Dr. Paula Davis	Cornell University
Dr. Richard Roush	Cornell University
Dr. Dennis Calvin	Penn State University
Dr. Galen Dively	University of Maryland
Dr. Ames Herbert	Virginia Polytechnic Institute
Dr. Fred Gould	North Carolina State University
Dr. John Van Duyn	North Carolina State University

Appendix IV:

Safety of Insect-Protected maize on non-target insects.

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Safety of Insect-Protected maize on non-target insects.

Studies were conducted in 1993, 1994 in the U.S. and in 1995 in France and in the U.S in order to compare inbreds and hybrids of Insect-Protected maize transformed with the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* with their non-transformed counterparts. During these studies the relative abundance of beneficial arthropods population between transformed and non-transformed maize and between CryIA(b) protection and insecticide sprays was evaluated.

(a) Impact of Insect-Protected maize on beneficial arthropods. Field studies in France in 1995

In 1995, two efficacy trials were conducted in France, at Virazeil and Pau, with hybrids progeny of Insect-Protected maize line MON 802 and MON 810. The purpose of these trials was to assess the insect control performance of these Insect-Protected maize lines, and compare the yield protection to their non transformed parent MON802/Bt- and MON810/Bt- protected by an insecticide spray. In order to ensure the development of European Corn Borer infestation and assess the efficacy of the *cryIA(b)* gene, half of the plots in the trials was inoculated with ECB. The trial was set-uo following a full factorial design with presence/absence of each of the three factors: Bt gene, ECB inoculation and insecticide spray (delta-methrine at 20g/ha).

In addition to these performance assessments, the number of beneficial arthropods was evaluated on ten plants in each plot of these trials. The evaluations took place in August 1995 and were done by Dr Kevin L. Steffey, Professor of Agricultural Entomology, University of Illinois, USA.

The figures a-1 and a-2 summarise the countings of the beneficial arthropods at both locations in August 1995. Each figure represents the total of beneficial arthropods present on 30 plants. These beneficial arthropods were generalist predators belonging to Anthocoridae (minute pirate bugs), Coccinellidae (lady beetles and other coccinellids), Nabidae (damsel bugs), Staphylinidae (rove beetles), lacewings and spiders.

Results

The comparisons between Insect-Protected maize MON802 and MON 810 and the non-transformed counterparts, MON802/Bt- and MON810/Bt-, in the absence of an insecticidal spray, show no significant difference in the beneficial arthropods populations at both sites Virazeil and Pau (figure a.1.).

At both sites, the comparisons between the plots receiving an insecticide spray and the non treated plots protected by the *cryIA(b)* gene clearly show the beneficial impact of the CryIA(b) insect protection with regard to the beneficial arthropods populations. The total populations of beneficial arthropods in the MON 810 and MON 802 plots were 3 to 4 time higher than in the insecticide protected plots (figure a.2.).

Discussion

Because the CryIA(b) protein produced by the Insect-Protected maize is not effective against the beneficial arthropods, beneficial populations are expected to rise significantly in maize fields planted with Insect-Protected maize.

Beneficial arthropods: average /30 plants (August 1995)

Monsanto Company confidential

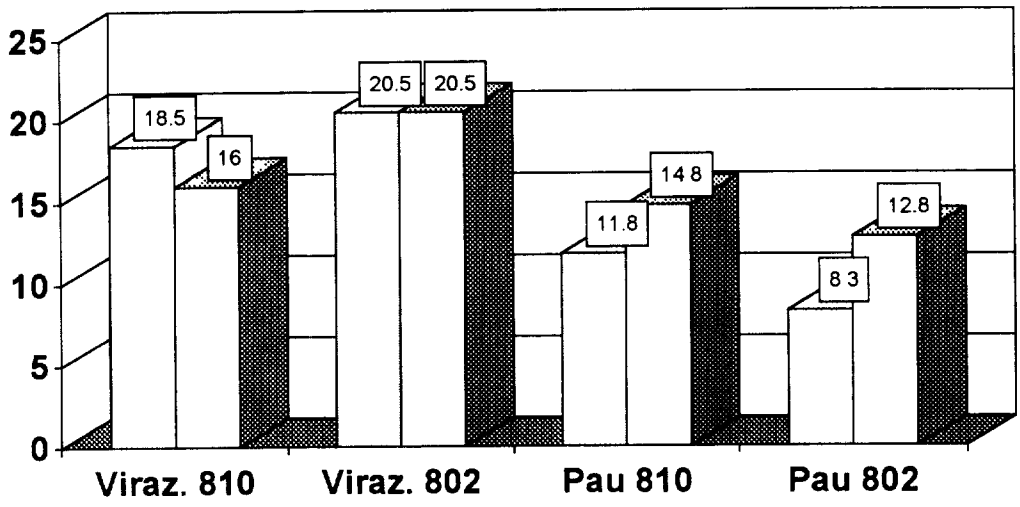

figure a.1.

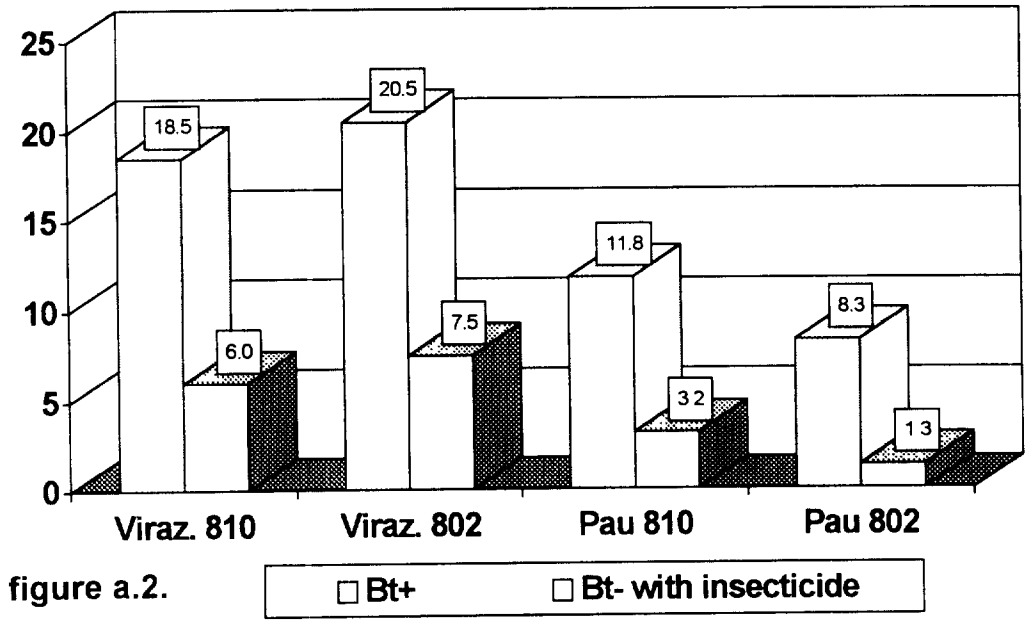

figure a.2.

(b) Impact of Insect-Protected maize on beneficial arthropods. Field and laboratory studies in 1993, 1994, and 1995.

Introduction

Field trials were conducted during 1993, 1994, and 1995 to assess the impact of insect-protected maize on beneficial arthropods. Maize inbreds and hybrids transformed with the *cryIA(b)* gene from *Bacillus thuringiensis* subsp. *kurstaki* were contrasted with their non-transformed counterparts for relative abundance of beneficial arthropods. A laboratory feeding studying was conducted in 1995 to assess the effect of pollen from insect-protected maize on two key predator species. Materials and methods employed, results, and discussion are provided.

Materials and Methods

1993

Two locations were sampled in 1993: Winterset, Iowa (IA) and York, Nebraska (NE), U.S.A. Each location had four inbred entries: two insect protected maize lines (MON 801 and maize line 523-06-1) and two non-transgenic inbreds, with eight rows of each/location.

Non-destructive visual sampling was conducted to assess the numbers of key beneficial arthropods. Plants were inspected for the presence of the insects of interest. Inspection on the part of the sampler was concentrated in the areas of the plant known to be occupied by the insects of interest. Counts were made on five plants per row on each sampling date. Time of sampling and growth stages were as follows for Winterset, IA: 15 July (whorl), 27 July (pre-anthesis), and 11 August (post-anthesis). For York, NE the dates and stages were: 20 July (pre-anthesis) and 9 August (post-anthesis).

1994

Two locations were sampled in 1994: Johnston (Harding) and Sheldahl, IA, U.S.A. Four transformation events were evaluated at both locations: maize line 523-06-1, maize line 546-09-1, MON 809, and MON 801. Twelve entries were evaluated at the Harding, IA location and eight at Sheldahl, IA. There were four replications per location of the following entries:

Harding, IA	Sheldahl, IA
Hybrid D	Hybrid G
Hybrid D/MON 801	Hybrid G /MON 801
Hybrid D /546-09-1	Hybrid G /546-09-1
Hybrid D /523-06-1	Hybrid G /523-06-1
	Hybrid H
Hybrid G/MON 801	Hybrid H /MON 801
Hybrid G /546-09-1	Hybrid H /546-09-1
Hybrid G /523-06-1	Hybrid H /MON 809
Hybrid H	
Hybrid H/MON 801	
Hybrid H /546-09-1	
Hybrid H /MON 809	

Sampling protocols were the same as those for 1994. Maize growth stages at time of sampling were as follows:

	5 July	27 July
Sheldahl, IA	V8-10	anthesis
Harding, IA	V8-10	anthesis

1995

The study sites in 1995 were located at Bonnut, France, Lexington, Kentucky (KY), Ames, Iowa (IA). The Bonnut location had a total of 60 entries, of which 52 were insect-protected and the remaining eight serving as non-transgenic controls. The insect-protected entries comprised four different transformation events: maize line 654-04-1, maize line 600-14-2, MON 810, and MON 801. Visual sampling was conducted on 12 August 1995 to determine the abundance of spiders and Anthocorids, with five plants per row serving as the sample unit.

The Lexington, KY location had a total of twelve entries replicated four times. Visual sampling was conducted three times: 11 July (whorl stage), 20 July (anthesis), and 7 August (post- anthesis). Five plants were sampled per row on each date. The events evaluated were maize line 654-04-1, MON 801, and MON 809. The actual entries were:

Lexington, KY
Hybrid I
Hybrid I /MON 801
Hybrid D
Hybrid D /MON 801
Hybrid J
Hybrid J /MON 809
Hybrid A
Hybrid A /654-04-1
Hybrid H
Hybrid H /MON 801
Inbred 07
Inbred 07/654-04-1

The Ames, IA site was established by Dr. Marlin E. Rice of Iowa State University utilizing MON 810 and non-transgenic control in a replicated plot design. Predators were counted at three times near anthesis: 1 August (before pollen shed), 6 August (during pollen shed), and 18 August (after pollen shed). Eighteen plants were marked, six per replication (four replications), within each treatment. The same plants were checked on each sampling date. The predators present consisted of coccinellid eggs, larvae, and adults, chrysopid eggs, larvae, and adults, nabids and arachnids. The number of European corn borer egg masses were also recorded.

A laboratory study was conducted in 1995 to measure the impact of pollen from insect-protected maize on the development and growth of two species of predators: an anthocorid - *Orius insidiosus* and a lady beetle - *Coleomegilla maculata*. In this study pollen collected from field grown Hybrid D and Hybrid D/MON 801 was fed to newly eclosed nymphs/larvae of the two predators. Insects were reared singly at 27 °C. They were provided water as a moisture source but only pollen as food. Developmental times and adult weights were assessed for predators fed pollen from the insect-protected verses non-transgenic hybrids.

The 1995 field and laboratory studies in Lexington both were conducted by Dr. Kenneth Yeargan, Department of Entomology, University of Kentucky, Lexington, Kentucky.

Results

1993

Results of the 1993 field sampling are presented in Table b-1. Adult *Orius insidiosus* was the only predator present in consistent abundance to evaluate. Values are the average number of *Orius* adults per five plants. Truly replicated designs were not employed and therefore no statistical analysis was conducted. The trend indicated no adverse effect on predator numbers due to the presence of the *cryIA(b)* gene.

Table b-1. *Orius* Adults in Winterset, IA and York, NE field locations (1993).

	Winterset, IA			York, NE	
	July 15	July 27	Aug. 11	July 15	July 27
Inbred #1	0.5	1.0	13.5	2.25	4.0
B73	2.0	3.5	20.75	5.0	5.0
B73/MON 801	3.25	4.25	19.25	6.75	6.0
B73/523-06-1	2.75	8.0	20	4.25	5.5

1994

Counts of *Orius* adults for 1994 are presented in Table b-2. A truly replicated design was used and statistical analysis was performed. There was only one instance of an insect-protected hybrid differing significantly from its non-transgenic counterpart for mean number of *Orius* adults. This was detected at Harding on 5 July for Hybrid D and Hybrid D/546-09-1. The insect-protected form of the hybrid showed the greater number of predators.

Table b-2. *Orius* Adults in Harding and Sheldahl, IA field locations (1994).

	Harding		Sheldahl	
	July 5	July 27	July 5	July 27
Hybrid H	11.25	42.25	29.0	7.5
Hybrid H/MON 809	9.25	31.25	37.0	8.0
Hybrid H/546-09-1	7.5	31.75	40.25	7.25
Hybrid H/MON 801	13.25	42.25	36.75	7.0
Hybrid G	9.75	42.5	53.5	6.25
Hybrid G/523-06-1	9.5	39.0	39.25	7.0
Hybrid G/546-09-1	10.25	36.0	48.5	3.75
Hybrid G/MON 801	7.75	37.75	45.25	7.25
Hybrid D	12.0	33.0	NP*	NP
Hybrid D/523-06-1	11.75	37.75	NP	NP
Hybrid D/546-09-1	17.25	41.0	NP	NP
Hybrid D/ MON 801	14.5	40.75	NP	NP
LSD	4.2	12.4	15.2	4.3

*NP: Not Planted at the Sheldahl, IA location

1995

Counts of spiders and anthocorids from the Bonnut, France field test are presented in Tables b-3 and b-4. Counts are from the averages for the first replication of the study. Comparisons on an average for insect-protected verses non-transgenic hybrids (Table b-3) and comparisons between the different events compared to the transgenic control (Table b-4) are shown.

Table b-3. Numbers of spiders and anthocorids in Bonnut, France field location (1995).

	Spiders	Anthocorids
Insect-Protected Maize	6.1	2.7
Non-transgenic Maize	6.5	2.9

Table b-4. Numbers of spiders and anthocorids on specific insect-protected maize lines in Bonnut, France field location (1995).

	Spiders	Anthocorids
Control	6.5	2.9
Maize line 654-04-1	4.5	3.3
Maize line 600-14-2	6.9	3.1
MON 810	7.0	2.0
MON 801	5.9	2.5

Insect counts were extremely low for both of the groups sampled. Comparisons made between all insect-protected hybrids and the controls indicated almost equal values for in the two groups: spiders as well as anthocorids (Table b-3).

When insect-protected hybrids were separated by insertion event and compared to the control, minor differences observed to occur for spiders as well as for anthocorids (Table b-4). However, differences were small and in some comparisons the insect-protected hybrids had a great number of beneficials as compared to the non-transgenic controls and for other comparisons the reverse was observed.

Table b-5 presents the mean number of *Orius* adults per plant on the three sampling dates at the Lexington location. There were only three comparisons between the insect-protected hybrid and its non-transgenic counterpart that resulted in a significant difference in mean number of *Orius* per plant. For two of these, the number of *Orius* per plant was higher for the transgenic and for one the number was less. Overall, the numbers were comparable.

Table b-5. Mean number of *Orius insidiosus* per plant in Lexington, KY (1995).

Hybrid	11 July	20 July	7 August
Hybrid I	5.1	8.5	2.5
Hybrid I/MON 801	4.7	12.0	2.8
Hybrid D	4.4	10.8	2.4
Hybrid D/MON 801	4.5	14.8	2.6
Hybrid J	6.6	17.4	2.8
Hybrid J/MON 809	7.0	13.4	3.3
Hybrid A	6.3	9.5	1.2
Hybrid A/654-04-1	8.6	10.6	2.3
Hybrid H	4.5	12.5	2.8
Hybrid H/MON 801	4.1	15.0	2.6
Inbred #1	6.1	8.8	2.7
Inbred #1/654-04-1	6.2	11.1	2.9
LSD	2.3	2.7	1.0

Table b-6 presents a summary of predator and European corn borer egg mass counts from the Ames, IA location. Differences were observed in the abundance of predators at each sampling time, but no differences were observed between insect-protected and the nontransgenic maize.

Table b- 6. Mean total numbers of predators and ECB egg masses per plant in Ames, IA (1995). (Values are expressed as number predators/ECB egg masses)

	1 August (<pollen shed)	6 August (pollen shed)	18 August (>pollen shed)
Non-transgenic	2.9/0.1	3.7/0.1	4.5/0.6
MON 810	3.2/0.1	4.9/0.3	6.2/0.8

Mean developmental times to the adult stage and adult fresh weights, as affected by pollen source, are presented in Table b-7 for both of the predators tested: *Orius insidiosus* and *Coleomegilla maculata*. Neither predator was seriously affected due to its feeding on pollen from an insect-protected hybrid as compared to a non-transgenic hybrid.

Table b-7. Mean developmental time to the adult stage and adult fresh weight for *Orius insidiosus* and *Coleomegilla maculata* fed pollen from insect-protected and non-transgenic maize hybrids. Lexington, Kentucky, 1995.

Pollen Source	N	Development Time (d)	Adult weight. (mg)
Coleomegilla maculata			
Hybrid D	30	18.1	12.1
Hybrid D/MON 801	28	17.8	11.2
Orius insidiosus			
Hybrid D	32	18.4	0.19
Hybrid D/MON 801	34	17.6	0.20

Discussion

Predators of the Family Anthocoridae, such as *O. insidiosus*, are of particular interest in studies to evaluate the potential impact of insect-protected maize plants on non-target insects. *Orius insidiosus* is one of the most abundant beneficial insects of maize in North America and is known to fed upon a wide spectrum of prey. One primary source of prey for *O. insidiosus* is the European corn borer, both the eggs and early instar larvae. This predator, like other anthocorids will utilize plant material (leaf, silks and pollen) to supplement its diet of arthropod prey. Therefore, these predators have the potential to be both indirectly as well as directly affected by a transgenic plant such as insect-protected maize. The indirect effects would be a reduction in prey availability due to the efficacy of the transgenic against the target pest. The direct effect would be the actual ingestion of the transgenic protein by the predators feeding upon the plant. The results observed during the three years reported herein shows neither a direct or an indirect effect on *O. insidiosus*.

In addition, spiders and other predators are known to be important predators of the European corn borer (*Ostrinia nubilalis*) as well as of other economically important pests of maize. Insect counts from Bonnut, France and Ames, IA in 1995 showed no effect on spiders, coccinellid, chrysopid and nabids at these sites attributed to the insect-protected corn hybrids .

Appendix II: CONFIDENTIAL BUSINESS INFORMATION

DNA Sequence of Plasmid PV-ZMBK07