Application for the Determination of Nonregulated Status for *B.t.* Cry34/35Ab1 Insect-Resistant, Glufosinate-Tolerant Corn: Corn Line 59122

CBI-DELETED COPY

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

Submitted by:

Penny L. Hunst, Ph.D.
Regulatory Manager,
Regulatory Success-Americas
Dow AgroSciences LLC
9330 Zionsville Road
Indianapolis, IN 46268
Telephone: 317-337-3977
Fax: 317-337-4649

Tracy Rood, B.S.
Registration Manager
Pioneer Hi-Bred International, Inc.
7250 NW 62nd Avenue
Box 552
Johnston, Iowa 50131
515-270-4036

December 18, 2003
REVISED: September 7, 2004

CLAIM OF DATA CONFIDENTIALITY

The Freedom of Information Act (FOIA) specifically exempts federal agencies from releasing information that are "trade secrets and commercial or financial information obtained from a person and privileged or confidential" 5 U.S.C. 552(b)(4). This exemption from FOIA's disclosure requirements for federal agencies applies where the disclosure of information would likely cause harm to the competitive position of the person from whom the information was obtained, or where, in the case of voluntarily submitted information, the submitter would be less likely in the future to voluntarily share data with the agency.

Appendix 2 of this Application for the Determination of Nonregulated Status for B.t. Cry34/35Ab1 Insect-Resistant, Glufosinate-Tolerant Corn: Corn Line 59122 is claimed by Dow AgroSciences LLC (DAS) as proprietary, trade secret and confidential information exempt from disclosure under FOIA. Appendix 2 contains proprietary information and research data that must be maintained as confidential as disclosure of this information would likely result in substantial competitive harm to DAS and provide a significant unfair competitive advantage to DAS' competitors. Disclosure of this information would allow DAS' competitors access to extensive proprietary research data which could enable such competitors to duplicate DAS' products or to develop competing products without incurring the millions of dollars and years of research and development expended by DAS. Disclosure of this information would also provide DAS' competitors with commercially valuable non-public information concerning DAS' product research, development and commercialization strategies, techniques, methods, operations, development timelines, etc. Keeping this information confidential is necessary for DAS to maintain its competitive position in a highly competitive high tech industry. DAS further desires to keep this information confidential to the extent patent applications have not been filed or patents are pending and have not been published. For these reasons, DAS claims the information in Appendix 2 is exempt from disclosure under FOIA.

Summary

Mycogen Seeds/Dow AgroSciences (Dow AgroSciences) and Pioneer Hi-Bred International, Inc. (Pioneer) are submitting a Petition for Determination of Nonregulated Status for *B.t.* Cry34/35Ab1 insect-resistant, glufosinate-tolerant corn line 59122. Dow AgroSciences and Pioneer request a determination from USDA - Animal and Plant Health Inspection Service (APHIS) that corn transformation event DAS-59122-7 and any corn lines derived from crosses with *B.t.* Cry34/35Ab1 corn line 59122 no longer be considered regulated articles under 7 CFR Part 340.

Dow AgroSciences and Pioneer have developed corn plants that contain a plant-incorporated-protectant (PIP) that effectively controls certain corn rootworm (CRW) pests. The tissues of these corn plants have been genetically modified, via recombinant DNA techniques, to express insecticidal crystal protein (ICP) from *Bacillus thuringiensis* strain PS149B1 which is selectively toxic to CRW. The Cry34/35Ab1 ICP consists of two proteins, approximately 14 kDa (Cry34Ab1) and 44 kDa (Cry35Ab1) in molecular weight. Transformation of these corn plants with the *cry*34Ab1 and *cry*35Ab1 genes resulted in the generation of event DAS-59122-7.

In addition to the insecticidal genes, the *pat* gene, which encodes the enzyme phosphinothricin acetyltransferase, is also present in event DAS-59122-7. The *pat* gene is a synthetic version based on the native *pat* gene from *Streptomyces viridochromogenes*, a non-pathogenic bacterium. The inclusion of the *pat* gene enables plant selection of the *Bt* lines and provides tolerance to glufosinate-ammonium herbicides. The PAT protein does not confer pesticidal activity and there are no known adverse environmental or toxicological effects.

The original DAS-59122-7 transformation event was produced by Pioneer using Mycogen's maize optimized (mo) *cry*34Ab1 and *cry*35Ab1 genes derived from the *Bacillus thuringiensis* strain PS149B1. *Bacillus thuringiensis*, a common Gram-positive soil bacterium, produces an insecticidal proteins that are very selective in toxicity to specific organisms. Decades of safety testing on *Bt* proteins demonstrate the lack of toxicity to humans and animals, and the absence of adverse effects on non-target organisms and the environment.

Corn line 59122 has been field tested since 2001 in the major corn growing regions of the United States as well as in Puerto Rico and Hawaii. All field tests have occurred under field notifications granted by USDA - APHIS. Information collected during those trials, laboratory analyses, reports and literature references presented herein demonstrate that line 59122 exhibits no plant pathogenic properties and is unlikely to harm other insects that are beneficial to agriculture. Corn line 59122 is no more likely to become a weed than non-transgenic corn and the *Bt* protein is unlikely to increase the weediness potential of any other cultivated plant or wild species. In summary, corn line 59122 is <u>not</u> likely to:

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

Dow AgroSciences and Pioneer request a determination from APHIS that *B.t.* Cry34/35Ab1 corn line 59122 and any progeny derived from crosses of this line with traditional corn lines, and any progeny derived from crosses of this line with transgenic corn lines that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR 340.

Certification

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

Penny L. Hunst, Ph.D.
Regulatory Manager

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

Telephone: 317-337-3977
Fax: 317-337-4649

Tracy Rood, B.S.
Registration Manager
Pioneer Hi-Bred International, Inc.
7250 NW 62nd Avenue
Box 552
Johnston, Iowa 50131

Telephone: 515-270-4036
Fax: 515-334-4478

TABLE OF CONTENTS

List of Tables

LIST OF FIGURES

List of Appendices

List of Attachments

ACRONYMS AND SCIENTIFIC TERMS

59122	Corn line containing event DAS-59122-7
ADF	Acid detergent fiber
ANOVA	Analysis of variance
APHIS	Animal and Plant Health Inspection Service, USDA
BCW	Black cutworm
bICP	Binary insecticidal crystal protein
bp	Base pair
B.t.	*Bacillus thuringiensis*
CaMV	Cauliflower mosaic virus
CaMV35S PRO	35S promoter from Cauliflower mosaic virus; 35S PRO
CaMV35S TERM	35S terminator from Cauliflower mosaic virus; 35S TERM
CRW	Corn rootworm
*cry*34Ab1	Gene encoding the maize-optimized Cry34Ab1 protein (or probe) from *Bacillus thuringiensis* strain PS149B1
*cry*35Ab1	Gene encoding the maize-optimized Cry35Ab1 protein (or probe) from *Bacillus thuringiensis* strain PS149B1
CFIA	Canadian Food Inspection Agency
CFSAN	Center for Food Safety and Nutrition, FDA
CLA	Corn leaf aphid
DAS	Mycogen Seeds c/o Dow AgroSciences LLC
DNA	Deoxyribonucleic acid
ECB	European corn borer
ELISA	Enzyme linked immunosorbent assay
EPA	Environmental Protection Agency (US)
Event DAS-59122-7	Corn event expressing the Cry34Ab1, Cry35Ab1 and PAT proteins
FDA	Food and Drug Administration (US)
FR	Federal Register (US)
Hi-II	Publicly available inbred corn line, the recipient corn line for event DAS-59122-7
ICP	Insecticidal crystal protein
IgG	Immunoglobulin antibody
Kb	Kilobase pair
kDa	Kilodalton, a measurement of protein molecular weight
MALDI-TOF MS	Matrix assisted laser desorption ionization – time of flight mass spectroscopy
MCRW	Mexican corn rootworm, *Diabrotica virgifera zeae*
nCRW	Northern corn rootworm, *Diabrotica berberi*
PAT	Phosphinothricin acetyltransferase protein encoded by the *pat* gene
PBN	US FDA Pre-market Biotechnology Notification
PHI	Pioneer Hi-Bred International, Inc.—A DuPont Company
Pf	*Pseudomonas fluorescens*
PHP17662	Plasmid construct containing the *cry*34Ab1, *cry*35Ab1 and *pat*

	genes and the genetic material necessary for their expression
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sCRW	Southern corn rootworm, *Diabrotica undecimpunctata howardii*
TA PEROXIDASE PRO	Promoter from *Triticum aestivum* peroxidase; bases 45-1342 from GenBank X53675 (Hertig *et al*., 1991); or wheat peroxidase promoter
UBI1ZM PRO	ubiquitin promoter (plus ubiquitin 5'UTR and intron) (Christensen *et al*., 1992) from *Zea mays*
USDA	United States Department of Agriculture
wCRW	Western corn rootworm, *Diabrotica virgifera virgifera*

I. RATIONALE

The commercial introduction of transgenic corn expressing the *Bacillus thuringiensis* (*B.t.)* Cry34/35Ab1 insecticidal crystal protein (ICP) will provide growers with a simple, cheap, highly effective, and environmentally benign means of controlling the western corn rootworm (*Diabrotica virgifera virgifera* LeConte; wCRW*)*,the northern corn rootworm (*Diabrotica barberi* Smith and Lawrence; nCRW*)* and the Mexican corn rootworm (*Diabrotica virgifera zeae* Krysan and Smith). Damage by corn rootworm currently costs growers $1 billion annually for treatment expenses and damages to the crop. Mycogen Seeds c/o Dow AgroSciences LLC (DAS) and Pioneer Hi-Bred International, Inc. (PHI) have developed *B.t.* Cry34/35Ab1 line 59122, which expresses the binary insecticidal crystal protein (bICP) composed of the Cry34Ab1 and Cry35Ab1 proteins, with efficacy against corn rootworm.

The Cry34Ab1 and Cry35Ab1 proteins were formerly known as the PS149B1 14 kDa and 44 kDa proteins. The proteins were given official Cry designations by the B.t. Nomenclature Committee in August, 2001. However, due to the fact that DAS and PHI had already begun regulatory work on the proteins, early permits and study reports refer to the ICP as the PS149B1 ICP or proteins.

Corn line 59122 (event DAS-59122-7) also contains the *pat* gene which encodes the enzyme phosphinthricin acetyltransferase (PAT). The *pat* is a synthetic version based on the native *pat* gene from *Streptomyces viridochromogenes*, a non-pathogenic bacterium. The inclusion of the *pat* gene enables plant selection of the *B.t.* lines and provides tolerance to glufosinate-ammonium herbicides. The PAT protein does not confer pesticidal activity, however, it does provide an alternative weed management tool to growers. *B.t.* Cry34/35Ab1 line 59122 may be used by growers as glufosinate-ammonium tolerant line. Glufosinate-ammonium has a history of safe use as a herbicide on corn in the U.S. and there are no known adverse environmental or toxicological effects.

II. THE CORN FAMILY (*ZEA MAYS* L.)

II.A. CORN AS A CROP

Corn is the major feed grain grown by farmers in the U.S., leading all other crops in value and volume of production. It is produced on every continent of the world with the exception of Antarctica. (Ohio Corn Marketing Program, 2000) (Table 1). Although corn is produced throughout the U.S., the major production area (accounting for over 50 percent of the corn grown) is located in the states of Iowa, Illinois, Nebraska and Minnesota. Other major corn growing states are Indiana, Ohio, Wisconsin, South Dakota, Michigan, Missouri, Kansas and Kentucky. All of these states together are collectively referred to as the “Corn Belt” (Ohio Corn Marketing Program, 2000).

The primary use of the corn produced each year is as an animal feed (50.1%). The remaining corn is exported (22.6%), held as ending stock (10.3%), processed into corn syrup for use as a sweetener (8.0%), converted into ethanol (5.0%), extracted for starch (2.6%), used in processed foods (1.2%) and grown as seed (0.2%) (Ohio Corn Marketing Program, 2000). The yellow dent type of corn is used for feed and this is grown as a commodity crop in North America. Wet milling to produce starch and sweetener products for foods accounts for the greatest volume of corn that is processed (Table 2). Non-food products such as industrial starches, corn gluten feed, and corn gluten meal are also manufactured (White and Pollak, 1995). The primary products derived from the dry milling process are corn grits, cornmeals, and corn flours. The largest food/feed product volume of the dry-milling industry is animal feed followed by brewing and food uses.

Table 1. Corn Production in Specified Countries[a]

	1999/00	2000/01	2001/02	2002/03
Country	Production			
Argentina	17,200[b]	15,400	14,700	15,500
Brazil	31,641	41,536	35,501	45,000
Canada	9,161	6,827	8,389	8,975
China, Peoples Republic of	128,086	106,000	114,088	121,300
European Union	36,404	37,823	40,006	40,089
Egypt	5,678	5,636	6,160	5,880
Hungary	7,000	5,000	7,600	6,000
Indonesia	6,200	5,900	6,000	6,100
India	11,470	12,068	13,510	11,100
Mexico	19,240	17,917	20,400	18,800
Nigeria	5,100	4,000	5,000	5,200
Romania	10,500	4,800	7,000	7,300
Philippines	4,449	4,508	4,505	4,300
South Africa, Republic of	11,455	8,040	10,050	9,200
Thailand	3,900	4,700	4,500	4,200
Ukraine	1,737	3,848	3,641	4,200
Others	58,601	52,719	56,814	60,495
Subtotal	**367,822**	**336,722**	**357,864**	**373,639**
United States	**239,549**	**251,854**	**241,485**	**228,805**
World Total	**607,371**	**588,576**	**599,349**	**602,444**

[a]USDA-FAS 2003. http://www.fas.usda.gov/psd/complete_tables/GF-table9-81.htm
[b]Numbers are 1,000 metric tons.

Table 2. Food and Industrial Corn Use: 1993-2002[a]

Crop Year[b]	HFCS[c]	Glucose & Dextrose	Starch	Fuel Alcohol	Beverage Alcohol	Cereals and Other Products	Total
2002	552[d]	215	255	925	131	187	2285
2001	541	217	246	714	131	186	2054
2000	530	218	247	628	130	185	1957
1999	540	222	251	566	130	185	1913
1998	530	219	240	526	127	184	1846
1997	513	229	246	481	133	182	1805
1996	492	233	238	429	130	172	1714
1995	473	227	226	396	125	161	1628
1994	459	224	230	533	100	150	1715
1993	441	219	225	458	110	140	1613

[a]Source: Feed Yearbook, USDA Economic Research Service, 2003.
http://www.ers.usda.gov/publications/so/view.asp?f=field/fds-bb/
[b]Crop year: September 1 – August 31.
[c]HFCS: High Fructose Corn Syrup.
[d]All numbers in the table are in million bushels.

II.B. TAXONOMY OF CORN

Family name: Poaceae (Gramineae)
Genus: *Zea*
Species: *mays* (2n = 20)

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

Teosinte is the common name for all the wild relatives of domesticated corn. Of the three taxa of teosinte, *Zea mays* spp. *Parviglumis* var. *parviglumis* is the closest living relative of corn (Iltis, 1988). Cultivated corn and the wild members of diploid and tetraploid *Zea* can be crossed to produce fertile F1 hybrids. However, in the wild, introgressive

hybridization does not occur because of differences in flowering time, geographic separation, block inheritance, developmental morphology and timing of reproductive structures, dissemination and dormancy (Galinat, 1988).

Tripsacum is also another close relative of domesticated corn, although it is a much more distant relative. Cultivated corn and all species of *Tripsacum* will hybridize, though F1 hybrids are highly male sterile. It has been suggested that Tripsacum and corn share a teosinte-like ancestor (Galinat, 1988).

The three species of *Tripsacum* that are native to North America are: *T. floridanum, T. lanceolatium, T. dactyloides. T. floridanum* is found in south Florida and is used as an ornamental grass for landscaping. *T. lanceolatim* is found in the Mule Mountains of Arizona and possibly in southern New Mexico. *T. dactyloides* (Eastern gamma grass) is indigenous to most of the southern, central and northeastern U.S. It is commonly grown as a forage grass and is the only species that overlaps with corn production areas (USDA, 1995; Hitchcock, 1971).

II.C. GENETICS OF CORN

The corn plant has separate male (staminate) and female (pistilate) flowering parts which encourages the natural outcrossing between corn plants. Open pollination of corn plants in the field leads to the production of grain that has the properties of many different lines and is no longer "true-breeding" (Canadian Food Inspection Agency, 1994; Aldrich, et al., 1986).

Corn hybrids are the first generation grown from seed produced by crossing unrelated parents (generally inbred lines or single crosses) (Bauman and Crane, 1985). Inbreds are created by self-pollination, i.e., pollen from the tassel is placed on silks of the same plant. Pure inbred lines that are uniform and transmit their characteristics consistently to their next generation are the result of six to seven generations of selfing. The genetic variation within progenies is reduced by half after each generation of selfing. Controlled cross-pollination of inbred lines from chosen genetic pools combines desired genetic traits in a hybrid and results in a yield increase. The inbred-hybrid concept with the resulting yield response is the basis of the modern corn hybrid industry (CFIA, 1994).

II.D. POLLINATION OF CORN

Corn is a wind-pollinated plant. The separate tassels and silk flowers encourage the natural outcrossing between corn plants. Typical of wind-pollinated plants, a large amount of redundant corn pollen is produced for each successful fertilization of an ovule on the ear (Kiesselbach, 1949). Wind movements across the corn field cause pollen from the tassel to fall on the silks of the same or adjoining plants. However, pollen of a given plant rarely fertilizes the silks of the same plant. Under field conditions, 97% or more of

the kernels produced by each plant are pollinated by other plants in the field (Aldrich, et al., 1986). Corn pollen measures about 0.1 mm in diameter and is the largest of any pollen normally disseminated by wind from a comparably low level of elevation.

Dispersal of corn pollen is influenced by its comparatively large size and rapid settling rate (Raynor *et al.*, 1972). The majority of pollen produced within a field, remains in the field due to its density. Most of the pollen from a corn plant settles to the ground within the area of the plant itself. Pollen concentration within a 60 meter radius from the source quickly decreases to less than 1% (Raynor, *et al.*, 1972). The concentration of pollen drops off rapidly a short distance from the field.

Pollen survival is highly dependent on relative humidity. Under optimal temperature and humidity conditions, pollen viability remains for about 30 minutes (CFIA, 1994). The pollen grain will start growth of the pollen tube down the silk channel within minutes of coming in contact with a silk. The pollen tube grows the length of the silk and enters the ovule in 12 to 28 hours (Aldrich, et al., 1986).

II.E. WEEDINESS OF CORN

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

II.F. CHARACTERISTICS OF NON-TRANSFORMED CULTIVAR

The corn germplasm utilized as the initial recipient of the added genes is a public line designated Hi-II. Hi-II is a derivative of the A188 and B73 inbred lines of corn which are publicly available inbred lines from the University of Minnesota and Iowa State University, respectively. Hi-II is approximately 50:50 of the two lines (Armstrong, et al., 1991). The material was developed to have a higher regeneration potential .

III. DESCRIPTION OF THE TRANSFORMATION SYSTEM

The PHP17662 transformation vector is a T-DNA vector carrying the transgenes (*cry*34Ab1, *cry*35Ab1, *pat*) for insertion into the plant genome. The *Agrobacterium* vector contained the spectinomycin and tetracycline resistance genes in the backbone and an origin of replication for *Agrobacterium.* The T-DNA region of PHP17662 contained

the elements in the following order: Right T-DNA border, corn ubiquitin promoter, *cry*34Ab1 gene, PINII terminator, TA peroxidase promoter, *cry*35Ab1, PINII terminator, 35S promoter, *pat* gene, 35S terminator, Left T-DNA border (Table 1).

Event DAS-59122-7 was generated using plasmid PHP17662 (Figure 1) via *Agrobacterium*-mediated transformation (Zupan and Zambryski, 1995; 1997). The corn recipient line used in the transformation was the public line designated Hi-II. Immature embryos of corn were aseptically removed from the developing caryopsis and treated with *A. tumefaciens* strain LBA4404 containing plasmid PHP17662 (Figure 1). Embryos and *Agrobacterium* were co-cultivated on solid culture medium and the embryos were subsequently transferred to fresh culture medium that contained antibiotics and the herbicide glufosinate-ammonium. The antibiotics killed any remaining *Agrobacterium*. The culture medium was stimulatory to maize somatic embryogenesis and was selective for those cells that contain the integrated *pat* gene. Therefore, callus that survived the herbicide proliferated and produced embryogenic tissue was presumably genetically transformed. The embryonic tissue was then manipulated to regenerate whole transgenic plants, which were transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the transgenes by PCR and to confirm the expression of the foreign protein by ELISA. Plants were then subjected to a whole plant bioassay using corn rootworm (CRW). Positive plants were crossed with an inbred line to obtain seed from the initially transformed plants. A number of lines were evaluated in the field which resulted in the selection of line 59122, based on its good agronomic characteristics and excellent resistance to corn rootworm.

Figure 1 is the plasmid map of construct PHP17662 with all the elements identified. Figure 2 is a schematic diagram of the T-DNA region of PHP17662 with several restriction enzyme sites indicated.

Figure 1. Schematic map of plasmid PHP17662 that was used for *Agrobacterium*-mediated transformation. . Plasmid map of PHP17662 indicating restriction enzyme sites for *Xho* I and coding regions for *cry*34Ab1, *cry*35Ab1, *pat* and vector backbone genes including *spc*, *tet*, and *vir*G. Probe locations for the five vector backbone probes; *spc*, *tet*, *vir*G, RB backbone, and LB backbone, are indicated within the circle region of the plasmid map. Locations for restriction enzyme sites in plasmid PHP17662 relative to the starting base pair (1) on the plasmid map are as follows with the underlined locations indicating sites within the T-DNA region:
***Bsa* I** - 2263 bp, 3431 bp, 5509 bp, 5541 bp, 7645 bp, 12787 bp, 16214 bp, 24443 bp, 26273 bp, and 29628 bp;

***Hin*d III** - 39 bp, 11102 bp, 12011 bp, 13132 bp, 14647 bp, 18252 bp, 18311 bp, and 25274 bp;

***Nco* I** – 19364 bp, 21279 bp, 23886 bp, 34138 bp, and 34666 bp;

***Sac* I** - 10 bp, 1290 bp, 1341 bp, 8321 bp, 21288 bp, 23229 bp, 23352 bp, and 25207 bp;

***Xho* I** – 16 bp, 1330 bp, 2230 bp, 2276 bp, 2913 bp, 10734 bp, and 19018 bp

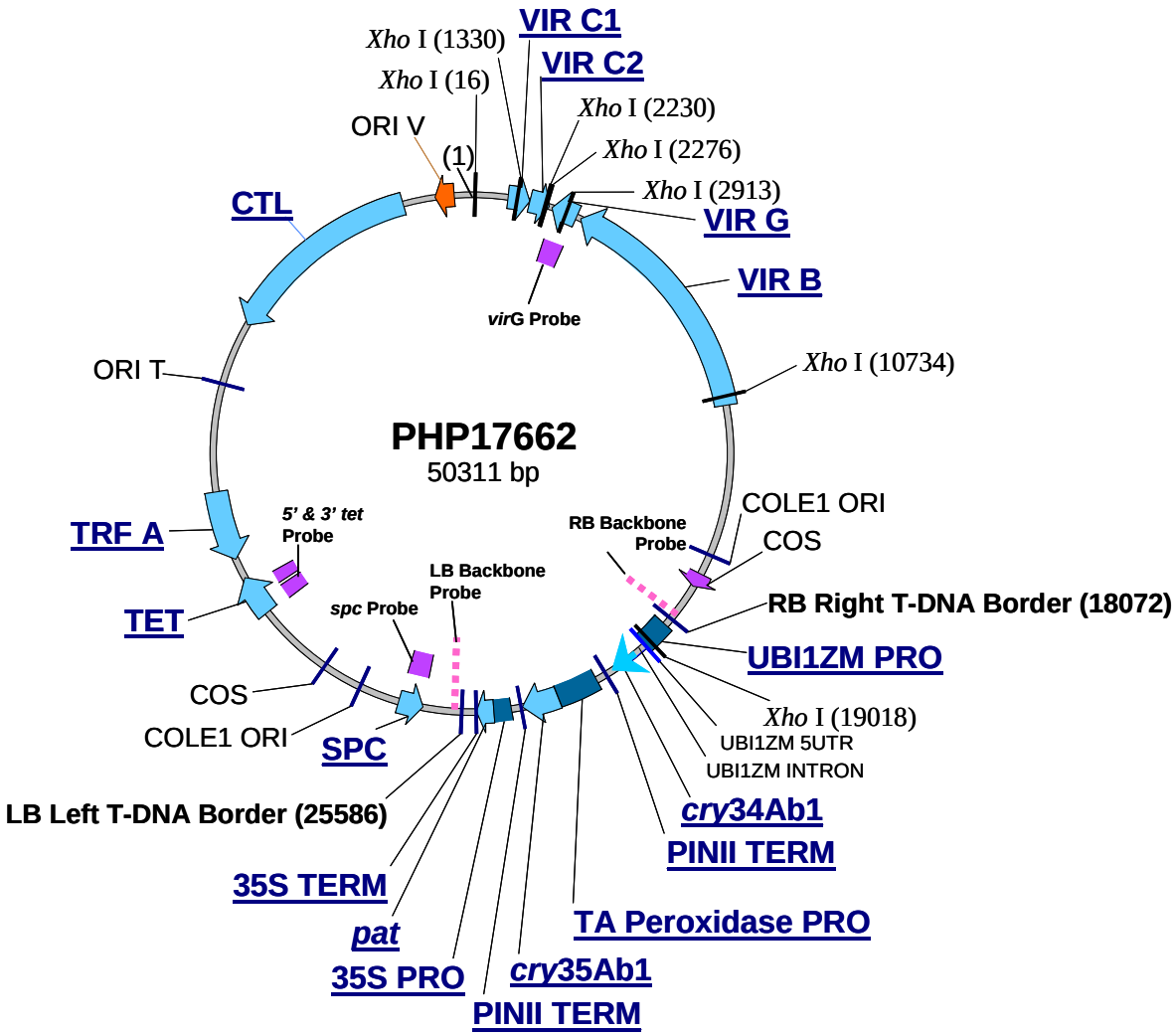
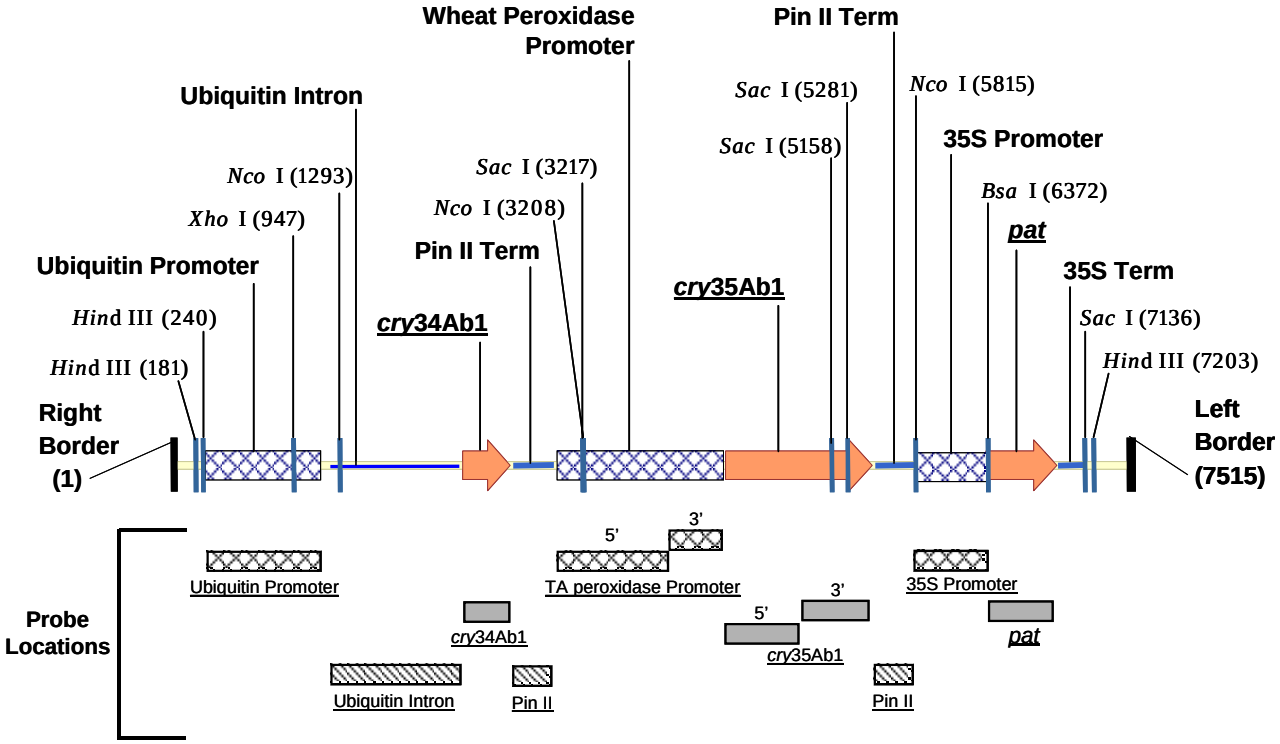


Figure 2. Schematic diagram of the T-DNA region of PHP17662 with restriction enzyme sites for *Bsa* I, *Hin*d III, *Nco* I, *Sac* I *and Xho* I. Schematic diagram of the T-DNA region from plasmid PHP17662 with gene elements underlined and other features in bold type. Restriction enzyme sites are given with bp positions. Probes used for Southern blot hybridization are indicated by boxes beneath the map. Actual length of the region from right border to left border is 7515 bp.



IV. THE DONOR GENES AND REGULATORY SEQUENCES

Event DAS-59122-7 was generated using plasmid PHP17662, which contains the synthetic, maize-optimized *cry*34Ab1, *cry*35Ab1 genes as well as the synthetic *pat* gene between the left and right borders of the *Agrobacterium* T-DNA. The *pat* gene is a selectable marker and also confers tolerance to glufosinate-ammonium herbicides. A summary of the genetic elements of PHP17662 is given in Table 3.

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

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

Since these early studies, numerous reports have demonstrated that more efficient nuclear expression of *B.t.* proteins in plants can be achieved using synthetic transgenes in which the relatively low G+C content of native bacterial δ-endotoxin genes is altered to more closely approximate the higher G+C content typically found in plant genes. Design of synthetic *B.t*. transgenes involves substitutions of alternative codons to bias the codon usage to that preferred by the target plant, and to remove certain deleterious A+T rich sequence elements responsible for mRNA destablization such as RNA cleavage signals, inappropriate polyadenylation sequences, intron splice sites, hairpin sequences, and transcription termination signals. Transgenes encoding Cry34Ab1 and Cry35Ab1 were optimized for corn expression using this general approach.

The *pat* gene is a synthetic version of the native bacterial *pat* gene sequence from *Streptomyces viridochromogenes* (Eckes *et al*., 1989.). The synthetic version was produced in order to modify the G+C codon bias to a level more typical for plant DNA. The synthetic, plant-optimized *pat* gene shows improved expression of PAT protein in corn plants. The promoter for the *pat* gene is the promoter of the 35S transcript from cauliflower mosaic virus (Pietrzak *et al*., 1986).

B.t. Cry34/35Ab1 corn line 59122 occurred as a simple integration of a single intact T-DNA from plasmid PHP17662. Digestion with *Xho* I, an enzyme that digests once in the T-DNA, produced one hybridizing band for all three gene probes, *cry*34Ab1, *cry*35Ab1, and *pat*, indicating one insertion in transgenic corn event DAS-59122-7 (Locke and Igo, 2003; Appendix 2, Section 6).

Table 3. Genetics elements of the plasmid PHP17662.

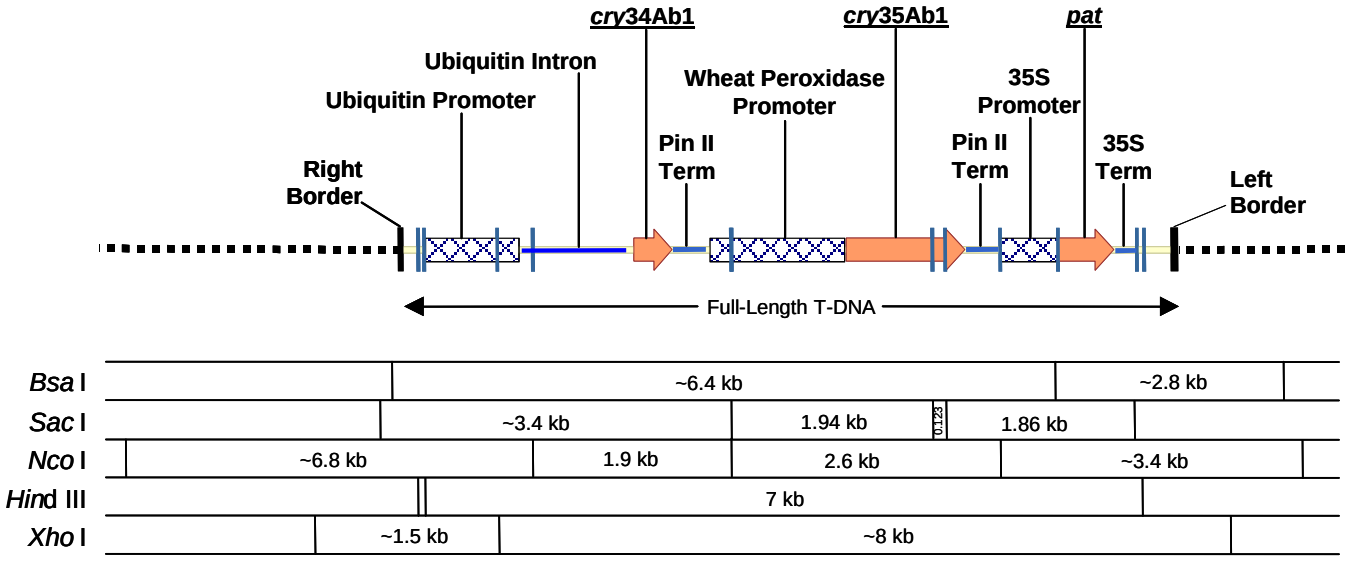
Genetic element	Size (bp)	Location on PHP17662 (bp)	Function
RB	25	18072-18096	Right Border
UBI1ZM PRO	1,986	18327-20312	ubiquitin promoter (plus ubiquitin 5'UTR and intron) (Christensen *et al*., 1992) from *Zea mays*
*cry*34Ab1	369	20342-20710	synthetic version of the *cry*34Ab1 gene encoding the 14 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 (NRRL B-21553)**]** of *Bacillus thuringiensis*
PINII TERM	318	20735-21052	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al*., 1989)
TA PEROXIDASE	1,299	21079-22377	promoter from *Triticum aestivum* peroxidase; bases 45-1342 from GenBank X53675 (Hertig *et al*., 1991)
*cry*35Ab1	1,152	22393-23544	synthetic version of the cry35Ab1 gene encoding a 44 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 (NRRL B-21553) of *Bacillus thuringiensis*
PINII TERM	318	23566-23883	terminator sequence from *Solanum tuberosum* proteinase inhibitor II (An *et al*., 1989)
CaMV35S PRO	549	23885-24433	35S promoter from Cauliflower Mosaic Virus, Strasbourg strain (Hohn *et al*., 1982)
PAT	552	24434-24985	synthetic, plant-optimized phosphinothricin acetyltransferase coding sequence from *Streptomyces viridochromogenes*
CaMV35S TERM	199	24998-25196	35S terminator from Cauliflower Mosaic Virus
LB	25	25439-25463	Left Border

V. GENETIC CHARACTERIZATION OF EVENT DAS-59122-7

Molecular characterization by Southern blot analysis concluded that the insert in *B.t.* Cry34/35Ab1 event DAS-59122-7 occurred as a simple integration of a single intact T-DNA from plasmid PHP17662. Two Southern blot studies were completed to determine copy number and intactness of the insert, determine the stability of the insert across multiple generations, and to provide a detailed restriction enzyme map of the insertion region. In the first study that compared two distinct generations of *B.t.* Cry34/35Ab1 event DAS-59122-7, digestion with *Xho* I, an enzyme that cuts once in the T-DNA, produced one hybridizing band for all three gene probes, *cry*34Ab1, *cry*35Ab1, and *pat,* indicating one T-DNA had inserted . Hybridization of *Sac* I digested DNA with the *cry*35Ab1 and *pat* gene probes resulted in bands of the expected size for an intact T-DNA insertion and hybridization with the *cry*34Ab1 gene probe produced one border fragment substantiating the *Xho* I results of a single T-DNA insertion. An additional digestion with *Hin*d III, an enzyme that releases all three gene transcription units as one DNA fragment, revealed that the T-DNA unit was inserted intact. Hybridization with five probes located outside of the T-DNA on plasmid PHP17662 did not detect any gene fragments, indicating the absence of the tetracycline and spectinomycin resistance genes, the *vir*G gene, and the absence of regions immediately adjacent to the Left and Right T-DNA Borders in transgenic corn event DAS-59122-7. Identical fragment sizes were observed in all cases for two distinct generations of transgenic corn event DAS-59122-7, indicating stability of inheritance across generations (Locke and Igo, 2003; Appendix 2, Section 6).

In the second study, a detailed restriction enzyme map of the insertion was hypothesized based on Southern blot analysis of the DNA inserted into event DAS-59122-7 (Figure 3). The Southern hybridization results indicated that a single, intact T-DNA inserted into the corn genomic DNA to produce event DAS-59122-7 and sites for *Bsa* I, *Nco* I, *Sac* I and *Xho* I were placed relative to the Right and Left Border regions of the T-DNA insertion. In addition, the results did not indicate that rearrangements of the T-DNA had occurred, as all internal restriction enzyme sites appeared to be intact and produced hybridizing fragments of the expected size (Locke *et al.,* 2003; Appendix 2, Section 7). Identical fragment sizes were observed in all cases for four distinct generations of *B.t.* Cry34/35Ab1 event DAS-59122-7 analyzed in the two studies, indicating stability of inheritance across generations.

Figure 3. Putative restriction fragment map of the DAS-59122-7 event insertion. A single copy of the T-DNA from PHP17662 is hypothesized to have inserted in the plant genome. The schematic map shows a horizontal dotted line representing plant genomic DNA and a schematic representing one full copy of the T-DNA. Below the schematic diagram are fragments and restriction enzyme sites from Southern blot hybridization experiments with 5 restriction enzymes. Sites found within the T-DNA region were confirmed from the Southern blots based on known sequence of the PHP17662 plasmid and sites within the plant genomic DNA were determined from Southern blot hybridization patterns.



V.A. SOUTHERN BLOT ANALYSIS

Southern hybridization was used to describe the integration pattern and determine copy number of the *cry*34Ab1, *cry*35Ab1, and *pat* genes and confirm the absence of tetracycline (*tet*) and spectinomycin (*spc*) genes and three other regions that are present outside of the T-DNA borders of the transformation vector (Locke and Igo, 2003; Appendix 2, Section 6). The integration of genetically modified material into a plant genome can occur at virtually any site in the plant genome. In the *Agrobacterium* transformation method used to produce *B.t.* Cry34/35Ab1 event DAS-59122-7, DNA located between the Left and Right Border sequences of the vector is transferred and inserted into the plant cell genome. The transferred DNA is called T-DNA and once inserted into the plant genome the genes on this segment of DNA that contain plant expression signals are recognized by the plant and can be expressed.

Seeds were obtained from two plant breeding generations of *B.t.* Cry34/35Ab1 event DAS- 59122-7, designated the T1S1 generation and the BC1 hybrid generation. The T1S1 generation seed consisted of the original Hi-II line containing event DAS-59122-7 (T0) crossed to elite inbred PH09B to give an F1 hybrid (T1), and then selfed to give T1S1 seed. The BC1 generation consisted of the first backcross generation of the T1 of

event DAS-59122-7 with the recurrent parent 05F. The BC1 generation was crossed to a second inbred, 581, to produce the planted hybrid seed. Plants of both generations were grown in growth chambers and leaf samples were obtained for genomic DNA extraction and analysis. Leaf samples of the unmodified control plants (Hi-II, P38, PH09B, 05Fx581), which contain a genetic background representative of the transgenic plants, were also obtained for DNA extraction and analysis. Positive and negative plants were confirmed by testing individual plants of the two segregating generations with lateral flow test strips specific for the Cry34Ab1 protein and leaf painting with the herbicide glufosinate-ammonium to detect the expression of the PAT protein.

Genomic DNA was isolated and analyzed from leaf samples from individual plants of the T1S1 and BC1 generations of *B.t.* Cry34/35Ab1 event DAS-59122-7 (4 plants expressing Cry34/35Ab1 and PAT and one null segregant per generation) and from at least one leaf sample of individual plants for each unmodified control line. The isolated genomic DNA from the unmodified control plants and event DAS-59122-7 plants was digested with appropriate restriction enzymes to analyze the insertion number and arrangement of the T-DNA from plasmid PHP17662. Positive controls consisting of unmodified control genomic DNA spiked with plasmid PHP17662 DNA at the indicated approximate gene copy equivalents were also digested with the appropriate restriction enzymes. Following digestion with the restriction enzymes, the fragments produced were electrophoretically separated by size through an agarose gel and transferred to nylon membranes. Southern hybridization was carried out using digoxigenin (DIG) labeled probes (Table 4 and Figures 1 and 2). Probes for the *cry*34Ab1 gene, the *cry*35Ab1 gene (combined 5' and 3' *cry*35Ab1 fragments homologous to this gene sequence), and the *pat* gene were used to detect the inserted transgenes. All DNA probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from fragments generated by primers specific to gene elements from plasmid PHP17662 or plasmids containing equivalent elements.

Table 4. Description of DNA probes used for Southern blot hybridization.

Probe Name	Genetic Element	Position on PHP17662 T-DNA (bp to bp)	Position on Plasmid PHP17662 (bp to bp)	Length (bp)
*cry*34Ab1	*cry*34Ab1 gene	2301 to 2617	20372 to 20688	317 bp
*cry*35Ab1	5' end of *cry*35Ab1 gene	4322 to 4788	22393 to 22859	467 bp
	3' end of *cry*35Ab1 gene	4893 to 5464	22964 to 23535	572 bp
pat	*pat* gene	6350 to 6897	24421 to 24968	548 bp
ubi pro	ubiquitin promoter	286 to 1143	18357 to 19214	858 bp
TA perox pro	5' end of wheat peroxidase promoter	3008 to 3900	21079 to 21971	893 bp
	3' end of wheat peroxidase promoter	3901 to 4298	21972 to 22369	398 bp
35S pro	35S promoter	5829 to 6346	23900 to 24417	518 bp
ubi intron	ubiquitin intron	1155 to 2235	19226 to 20306	1081 bp
PINII term	pinII terminator	2719 to 2952 and 5550 to 5783	20790 to 21023 and 23621 to 23854	234 bp
spc	Spectinomycin resistance gene	not applicable	26643 to 27417	775 bp
tet	5' end of tetracycline resistance gene	not applicable	32496 to33034	539 bp
	3' end of tetracycline resistance gene	not applicable	33140 to 33597	458 bp
LB backbone	Region immediately outside of the left T-DNA border	not applicable	25488 to 25833	346 bp
RB backbone	Region immediately outside of the right T-DNA border	not applicable	17638 to 17902	265 bp
*vir*G	*vir*G gene	not applicable	2496 to 3239	744 bp

Analysis of Integration Number for the cry34Ab1, cry35Ab1 and pat Genes

DNA samples from transgenic corn event DAS-59122-7, T1S1 and BC1 generations (4 DNA samples from plants expressing Cry34Ab1 and PAT and one null segregant per each generation), were cleaved with the restriction enzymes *Xho* I and *Sac* I to determine the number of insertions of the *cry*34Ab1, *cry*35Ab1, and *pat* genes into the corn genome (Locke and Igo, 2003; Appendix 2). The *Xho* I enzyme has one cleavage site located within the T-DNA region of plasmid PHP17662 (Figure 1 and Figure 2) and any hybridizing DNA fragment on the Southern blot with any of the three gene probes would be expected to result from digestion of the one site within the T-DNA and another site in the corn genome, flanking the Left Border of the T-DNA insert. Similarly, the *cry*34Ab1 gene probe when hybridized to *Sac* I digested DNA would detect a fragment produced by digestion of the *Sac* I site within the T-DNA located 3' to the *cry*34Ab1 transcription unit in the wheat peroxidase promoter (Figure 2; *Sac* I site at bp 3217) and a second site in the corn genome region flanking the Right Border of the T-DNA insert. Since the site in the corn genome flanking the insert is unique to the site of insertion, each independent insertion of the T-DNA will have a unique fragment, generally termed a border fragment. The number of bands produced from the enzyme digestion is directly related to the integration complexity, i.e., the greater the number of bands the more integrations that have occurred in the corn genome. In general, for T-DNA insertions, one hybridizing band produced from an enzyme that only cleaves once in the insert and outside of the probe region, usually indicates the presence of one copy of the T-DNA inserted at a single locus in the genome. Border fragments formed from the insertion of a full length T-DNA are typically larger than the predicted size from the T-DNA sequence due to the inclusion of genomic DNA in the fragment. The exact size of border fragments cannot be predicted in advance due to the unknown location of the cleavage site in the corn genome.

Southern blot hybridization results from a blot containing DNA digested with *Xho* I and hybridized with the *cry*34Ab1, *cry*35Ab1, and *pat* gene probes are shown in Figure 4, Figure 5 and Figure 6, respectively. The hybridizing band sizes detected on the Southern blots are reported in Table 5 along with the expected hybridizing fragment sizes based on the original T-DNA sequence from plasmid PHP17662. Hybridization of the *Xho* I blot with either the *cry*34Ab1, *cry*35Ab1, or the *pat* probe to DNA from T1S1 and BC2 plants of event DAS-59122-7 resulted in one hybridizing band with an approximate size of 8.0 kb. Detection of one single border fragment containing the T-DNA Left Border region with the *Xho* I digest and the three gene probes strongly suggested that a single T-DNA insert was present in transgenic corn event DAS-59122-7. Hybridization of *Sac* I digested DNA with the *cry*34Ab1 probe (Figure 7) also indicated one hybridizing border fragment of approximately 3.4 kb for the T-DNA Right Border region substantiating the *Xho* I data of one single T-DNA insert. Hybridizing *Xho* I and *Sac* I fragments containing the T-DNA Left Border and Right Border regions, respectively, were larger than predicted from the T-DNA sequence suggesting that an intact T-DNA was inserted. The individual T1S1 and BC1 plants produced identical hybridizing border fragments which are unique to the site of insertion for all probe and digest combinations revealing stability of the insert within and across the two generations analyzed. As expected, null

segregants of transgenic corn event DAS-59122-7 and control corn lines did not hybridize to either probe.

The location of the *Sac* I and *Xho* I restriction enzyme sites in the corn genomic DNA relative to the T-DNA insertion in event DAS-59122-7are shown in Figure 3.

Table 5. Predicted and observed hybridizing bands on Southern blots of transgenic corn event DAS-59122-7 probed with *cry*34Ab1, *cry*35Ab1, and *pat* probes.

Probe	Restriction Enzyme	Figure	Predicted Fragment Size from T-DNA[1] (bp)	Predicted Fragment Size from Plasmid PHP17662[1] (bp)	Observed Fragment Size (bp)[2]
*cry*34Ab1	*Hin*d III	8	6963	6963	6963[3]
*cry*34Ab1	*Sac* I	7	> 3217	12967	~ 3400
*cry*34Ab1	*Xho* I	4	> 6569	31309	~ 8000
*cry*35Ab1	*Hin*d III	9	6963	6963	6963[3]
*cry*35Ab1	*Sac* I	11	1941 1855 123[4]	1941 1855 123[4]	1941[3] 1855
*cry*35Ab1	*Xho* I	5	> 6569	31309	~ 8000
pat	*Hin*d III	10	6963	6963	6963[3]
pat	*Sac* I	12	1855	1855	1855[3]
pat	*Xho* I	6	> 6569	31309	~ 8000

[1] Predicted fragment sizes are based on the plasmid map and the T-DNA map of PHP17662 as shown in Figures 1 and 2, respectively.
[2] Observed fragment sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments on the Southern blots. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.
[3] Observed size of fragment or fragments is presumed to be the same as predicted due to equivalent migration with hybridizing bands in the plasmid (PHP17662) positive control lanes.
[4] The predicted 123 bp fragment was not detected, as fragments below approximately 1.0 kb were run off the gel during electrophoresis and were not transferred to the nylon membrane.

Figure 4. Southern blot analysis of DAS-59122-7; Xho I digest, cry34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Xho I and probed with the cry34Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Xho I
2	Hi II + 1 copy PHP17662	Xho I
3	DIG VII + ϕx	
4	05Fx581	Xho I
5	DAS-59122-7-T52 (s) T1S1	Xho I
6	DAS-59122-7-T46 T1S1	Xho I
7	DAS-59122-7-T49 T1S1	Xho I
8	DAS-59122-7-T55 T1S1	Xho I
9	DAS-59122-7-T59 T1S1	Xho I
10		

Lane	Sample	Enzyme
11	DAS-59122-7- T26(s) BC1	Xho I
12	DAS-59122-7- T22 BC1	Xho I
13	DAS-59122-7- T23 BC1	Xho I
14	DAS-59122-7- T24 BC1	Xho I
15	DAS-59122-7- T28 BC1	Xho I
16	P38	Xho I
17	PH09B	Xho I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Xho I
20	P38 + 3 copies PHP17662	Xho I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

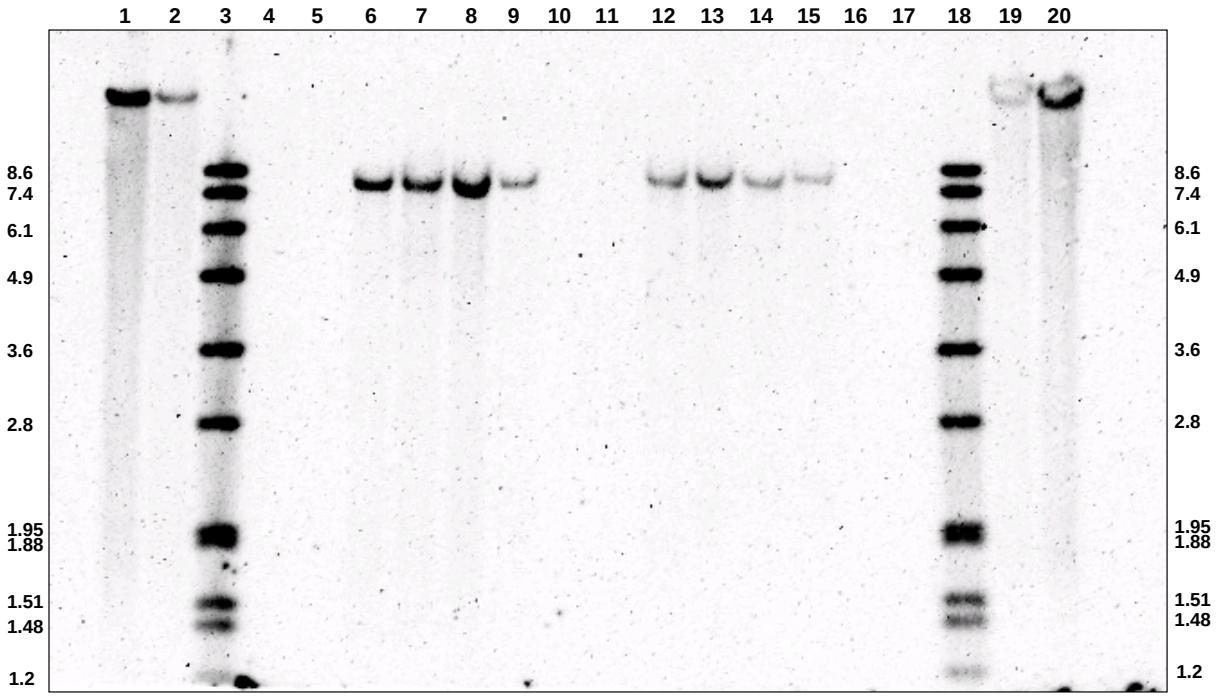


Figure 5. Southern blot analysis of DAS-59122-7; Xho I digest, cry35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Xho I and probed with the cry35Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi-II + 3 copies PHP17662	Xho I
2	Hi-II + 1 copy PHP17662	Xho I
3	DIG VII + ϕx	
4	05Fx581	Xho I
5	DAS-59122-7-T52 (s) T1S1	Xho I
6	DAS-59122-7-T46 T1S1	Xho I
7	DAS-59122-7-T49 T1S1	Xho I
8	DAS-59122-7-T55 T1S1	Xho I
9	DAS-59122-7-T59 T1S1	Xho I
10		

Lane	Sample	Enzyme
11	DAS-59122-7- T26(s) BC1	Xho I
12	DAS-59122-7- T22 BC1	Xho I
13	DAS-59122-7- T23 BC1	Xho I
14	DAS-59122-7- T24 BC1	Xho I
15	DAS-59122-7- T28 BC1	Xho I
16	P38	Xho I
17	PH09B	Xho I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Xho I
20	P38 + 3 copies PHP17662	Xho I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

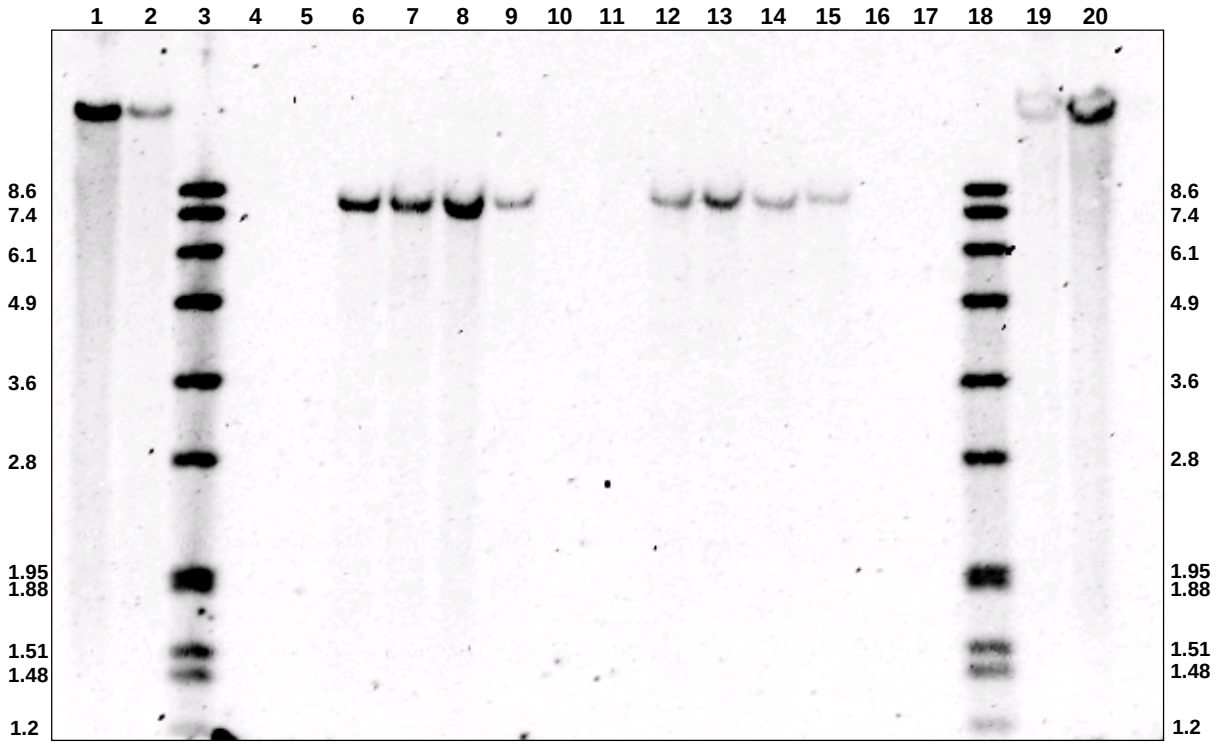


Figure 6. Southern blot analysis of DAS-59122-7; *Xho* I digest, *pat* probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with *Xho* I and probed with the *pat* gene probe. Approximately 7µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Xho I
2	Hi II + 1 copy PHP17662	Xho I
3	DIG VII + ϕx	
4	05Fx581	Xho I
5	DAS-59122-7-T52 (s) T1S1	Xho I
6	DAS-59122-7-T46 T1S1	Xho I
7	DAS-59122-7-T49 T1S1	Xho I
8	DAS-59122-7-T55 T1S1	Xho I
9	DAS-59122-7-T59 T1S1	Xho I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26(s) BC1	Xho I
12	DAS-59122-7- T22 BC1	Xho I
13	DAS-59122-7- T23 BC1	Xho I
14	DAS-59122-7- T24 BC1	Xho I
15	DAS-59122-7- T28 BC1	Xho I
16	P38	Xho I
17	PH09B	Xho I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Xho I
20	P38 + 3 copies PHP17662	Xho I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

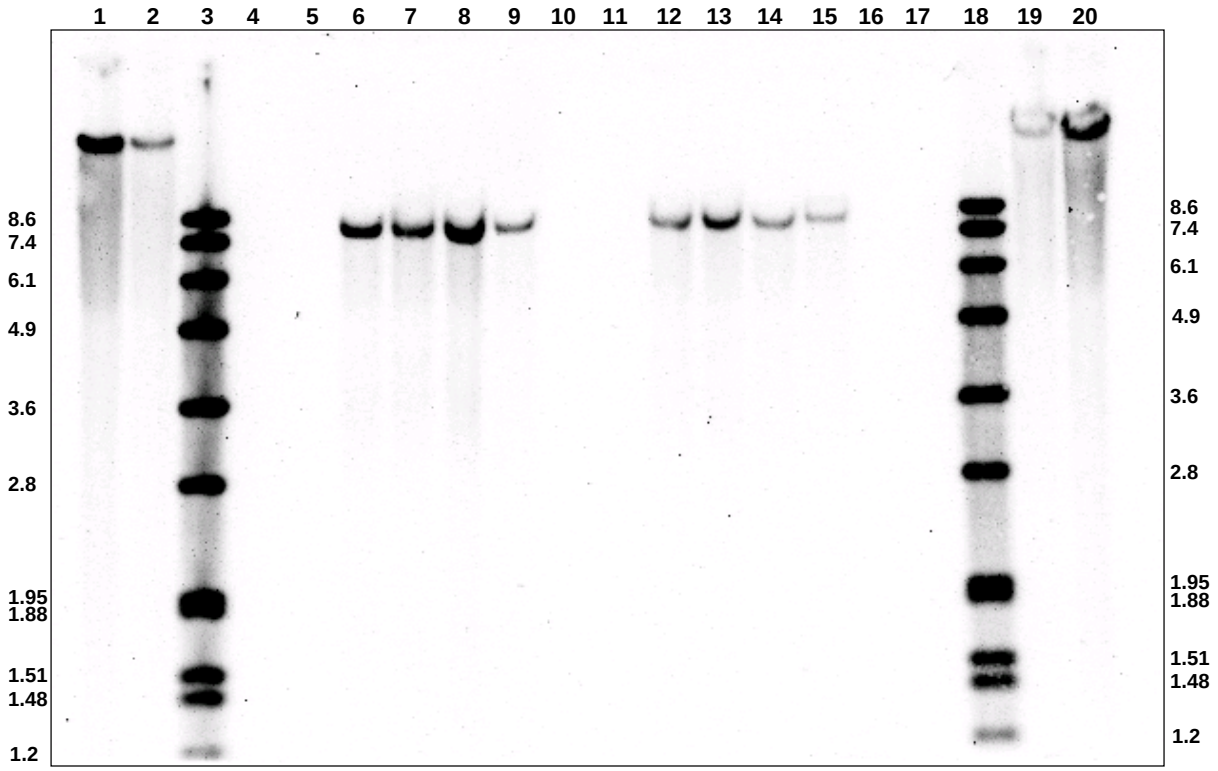
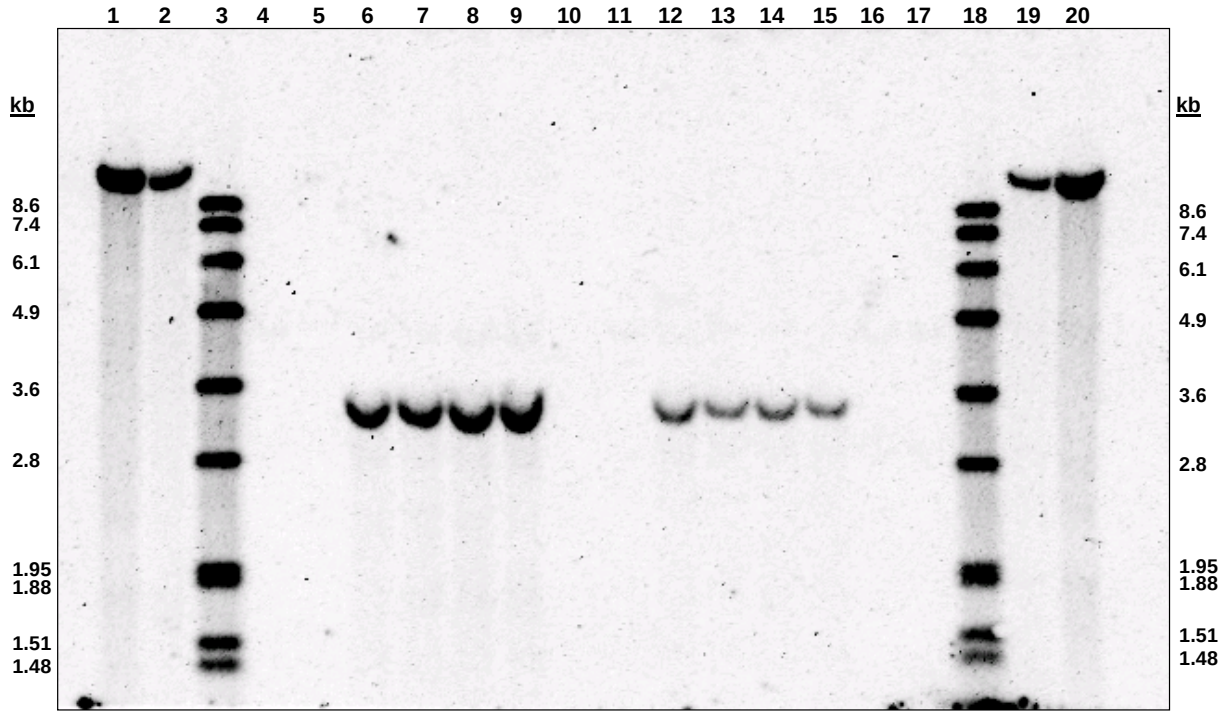


Figure 7. Southern blot analysis of DAS-59122-7; Sac I digest, cry34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the cry34Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. Note: A faint band of approximately 4.8 kb was visible across all test and control sample lanes. This band was not specific to the DAS-59122-7 event but was due to weak cross reactivity of the cry34Ab1 probe to corn genomic DNA or to DNA contained within the Sac I enzyme preparation.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.



Analysis of the Integrity of the Insertion for the cry34Ab1, cry35Ab1, and pat Genes

To confirm the integrity of the three genes within the inserted T-DNA, DNA from transgenic corn event DAS-59122-7, T1S1 and BC1 generations (DNA samples from 4 plants expressing Cry34Ab1 and PAT and one null segregant per each generation), was digested with *Hin*d III and hybridized to the *cry*34Ab1, *cry*35Ab1, and *pat* probes (Locke and Igo, 2003; Appendix 2, Section 6). In addition, the integrity of the *cry*35Ab1 and *pat* genes was assessed by hybridization of the respective gene probes to DNA from event DAS-59122-7 digested with *Sac* I. Along with the set of event DAS-59122-7 samples, positive controls consisting of unmodified control corn DNA spiked with plasmid PHP17662 DNA at approximately one and three gene copy equivalents were digested with *Hin*d III and *Sac* I. This allowed for visualization on the Southern blot of the predicted size fragments. Negative controls of unmodified corn DNA were also digested and analyzed.

The T-DNA diagram in Figure 2 outlines the *cry*34Ab1, *cry*35Ab1, and *pat* gene and probe locations and the *Hin*d III and *Sac* I restriction enzyme sites. The resulting fragments expected from the *Hin*d III and *Sac* I restriction digests based upon the T-DNA sequence are shown in Table 5. Digestion with *Hin*d III releases one fragment of 6.963 kb from the T-DNA (Figure 2) that is predicted to hybridize to the three gene probes: *cry*34Ab1, *cry*35Ab1, and *pat*. The *Hin*d III fragment contains the complete plant transcription units (promoter/gene/terminator) for all three genes. The *Sac* I restriction enzyme has four cleavage sites within the T-DNA region of plasmid PHP17662 (Figure 2) that result in internal hybridizing fragments of predicted size for the *cry*35Ab1 (1.941, 1.855, and 0.123 kb) and *pat* (1.855 kb) gene probes and a border fragment with genomic DNA that will hybridize with the *cry*34Ab1 gene probe. The predicted hybridizing fragment sizes and the observed hybridizing fragment sizes are listed in Table 4 along with the associated Southern blot Figure number.

As shown in Figures 8 - 10, the *cry*34Ab1, *cry*35Ab1, and *pat* gene probes, respectively, hybridized to a single *Hin*d III fragment of 6.963 kb in transgenic corn event DAS-59122-7 plants expressing both Cry34Ab1 and PAT for the T1S1 and BC1 generations as predicted for an intact T-DNA insert and as observed in the plasmid control lanes. Southern blots containing DNA from event DAS-59122-7 digested with *Sac* I and hybridized with the *cry*35Ab1 and *pat* gene probes are shown in Figure 11 and Figure 12, respectively. Hybridization of the *Sac* I blot with the *cry*35Ab1 probe resulted in two hybridizing bands of predicted size based on the intact T-DNA sequence; a strongly hybridizing band of 1.941 kb and weakly hybridizing band of 1.855 kb. The 1.941 kb band hybridized strongly to the *cry*35Ab1 probe since the probe overlaps with the majority of the fragment, whereas the weakly hybridizing 1.855 kb fragment only overlapped with 189 bp of the *cry*35Ab1 probe resulting in reduced signal intensity. The predicted 123 bp fragment was not detected, as fragments below approximately 1.0 kb were run off the gel during electrophoresis and were not transferred to the nylon membrane. Hybridization of the *Sac* I blot with the *pat* probe resulted in the predicted hybridizing fragment of 1.855 kb (Figure 12). Results of hybridization of the *Sac* I blot

with the *cry*34Ab1 probe were discussed in the previous section. Both the *cry*35Ab1 and the *pat* probe results on *Sac* I digested DNA produced the predicted hybridizing DNA fragments in *B.t.* Cry34/35Ab1 event DAS-59122-7 for both the T1S1 and BC1 generations, thus confirming the *Hin*d III results of an intact T-DNA insert. As expected, null segregants of event DAS-59122-7 and unmodified control corn lines did not hybridize to any of the three gene probes.

A summary diagram of the DNA insertion in transgenic corn event DAS-59122-7 is shown in Figure 3 and includes the summary for the restriction enzyme sites discussed above; *Hind* III, *Sac* I, and *Xho* I.

Figure 8. Southern blot analysis of DAS-59122-7; Hind III digest, cry34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the cry34Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7- T52 (s) T1S1	Hind III
6	DAS-59122-7- T46 T1S1	Hind III
7	DAS-59122-7- T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

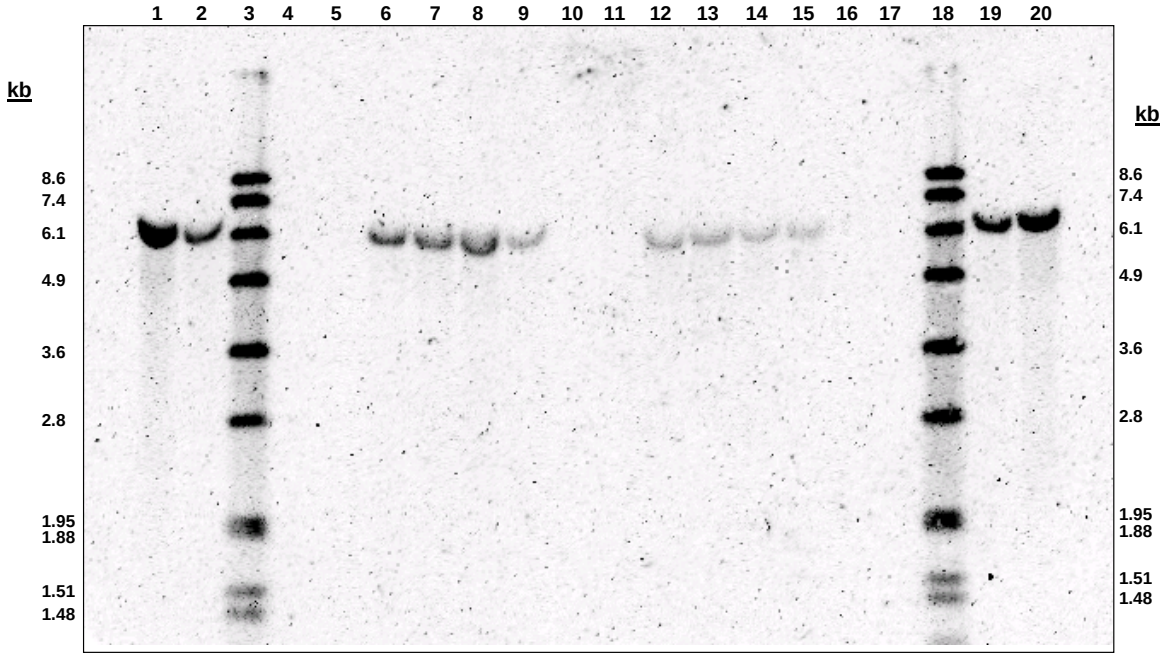


Figure 9. Southern blot analysis of DAS-59122-7; Hind III digest, cry35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the cry35Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7-T52 (s) T1S1	Hind III
6	DAS-59122-7-T46 T1S1	Hind III
7	DAS-59122-7-T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

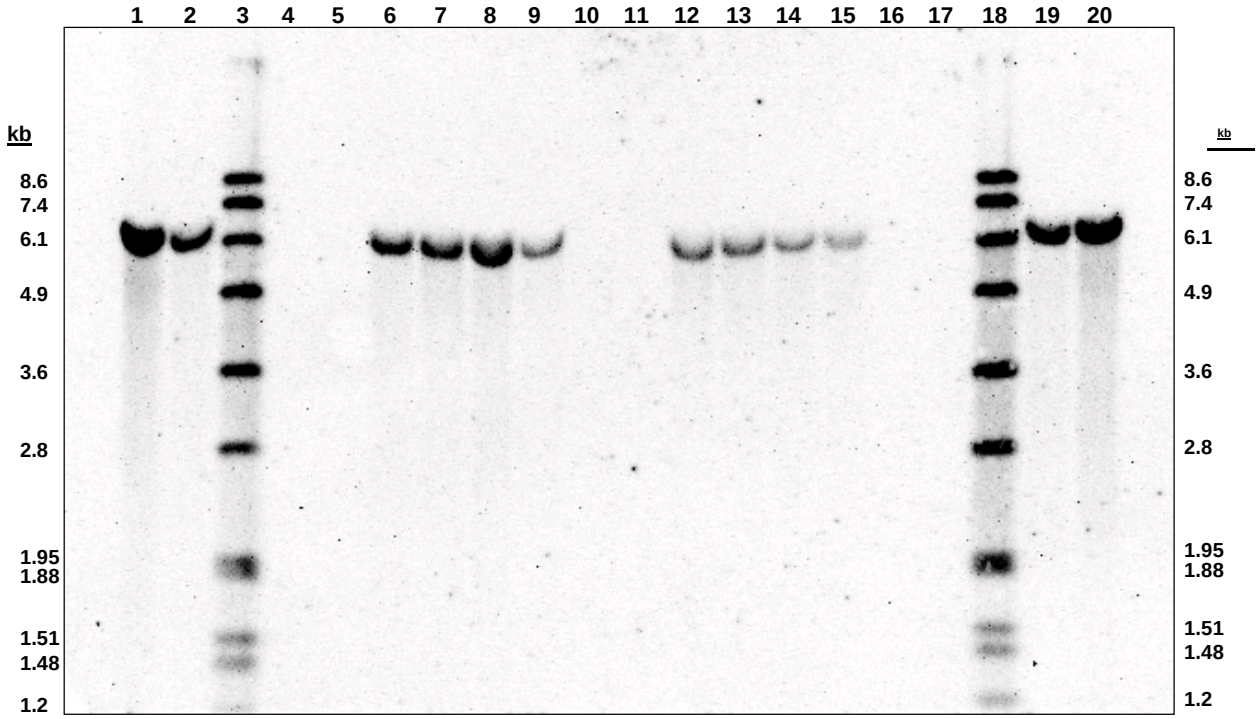


Figure 10. Southern blot analysis of DAS-59122-7; Hind III digest, pat probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the pat gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7-T52 (s) T1S1	Hind III
6	DAS-59122-7-T46 T1S1	Hind III
7	DAS-59122-7-T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

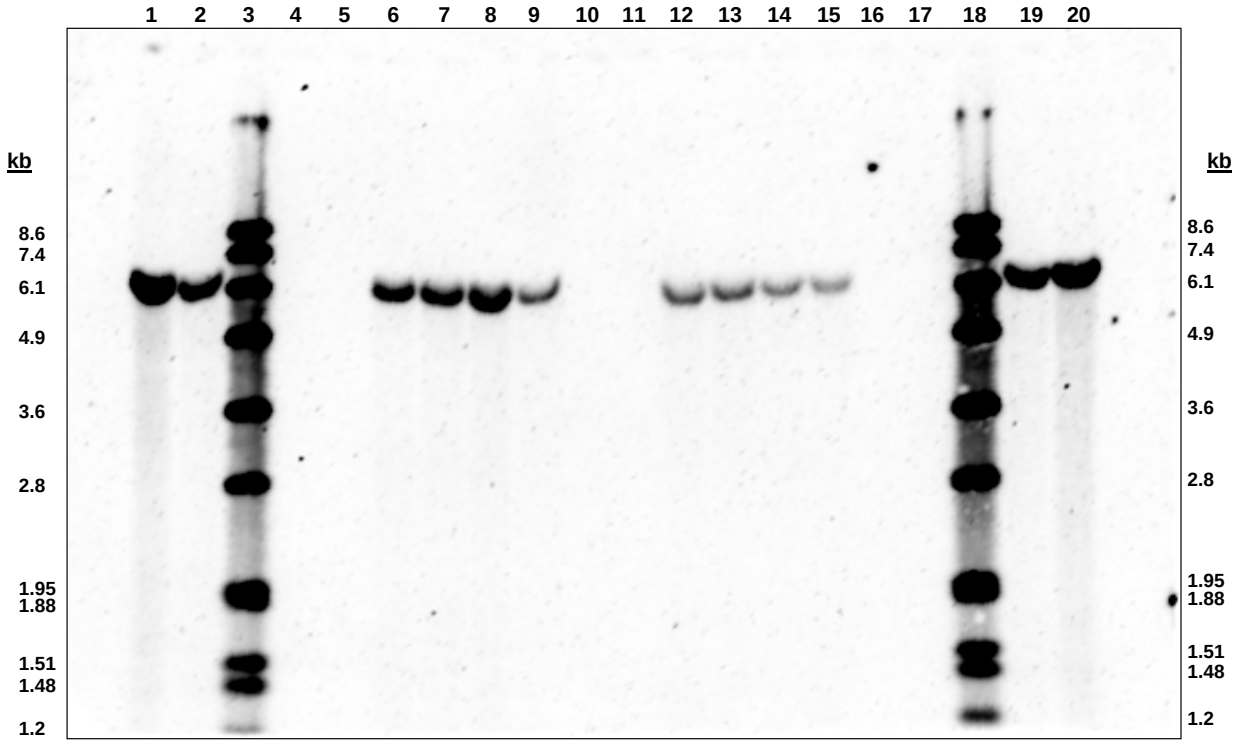


Figure 11. Southern blot analysis of DAS-59122-7; Sac I digest, cry35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the cry35Ab1 gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10		

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

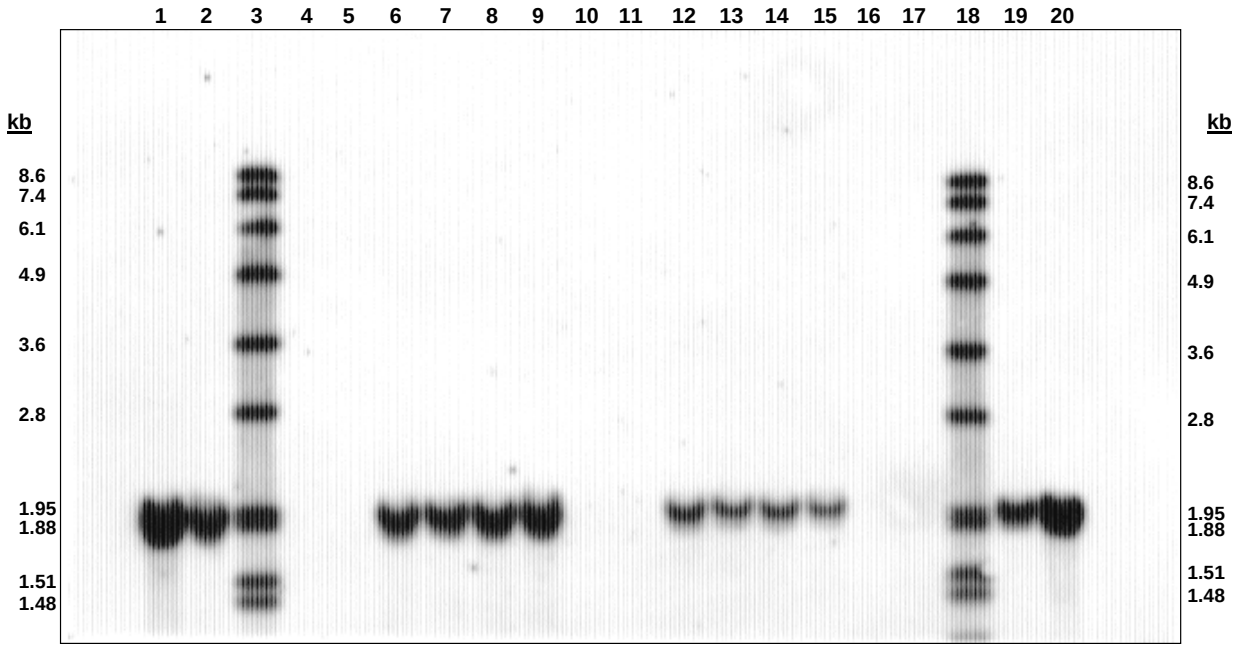
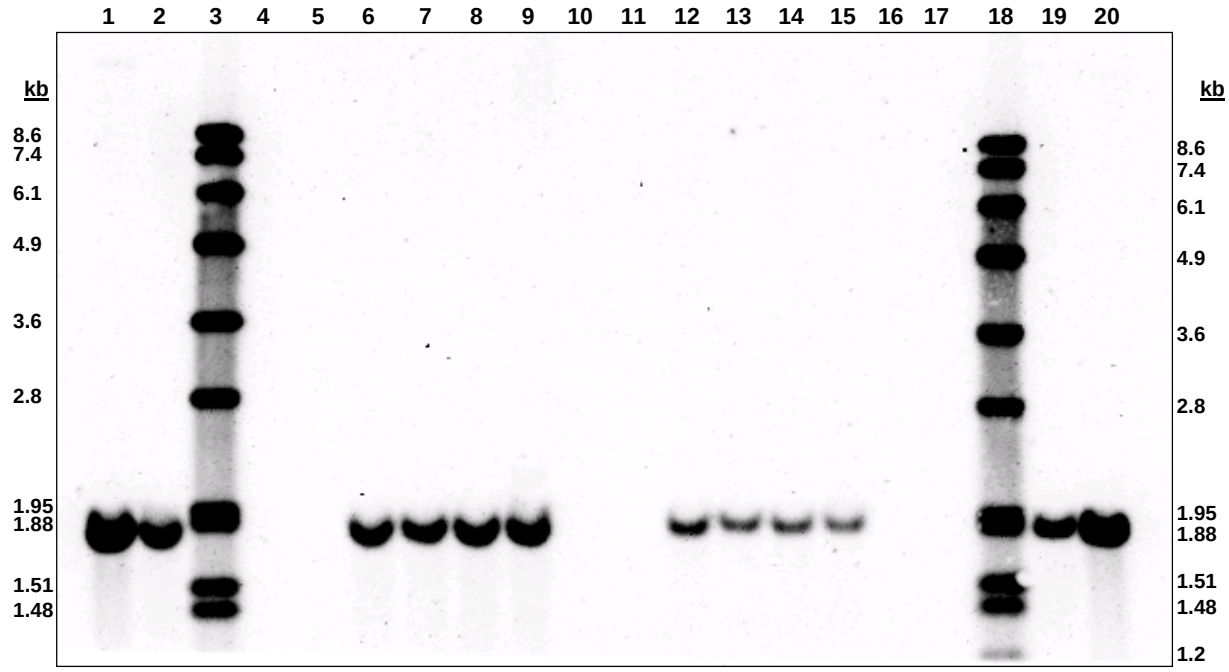


Figure 12. Southern blot analysis of DAS-59122-7; Sac I digest, pat probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the pat gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10		

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.



A detailed Southern blot characterization of the inserted DNA in transgenic corn event DAS-59122-7 that contains the *cry*34Ab1, *cry*35Ab1, and *pat* genes controlled by appropriate plant promoters and terminators was also conducted (Locke *et al.,* 2003; Appendix 2, Section 7). The integration pattern was investigated using selected restriction enzyme digest and probe combinations to characterize the inserted genes (*cry*34Ab1, *cry*35Ab1, and *pat* genes) and noncoding regions including promoters and terminators of gene expression.

Seeds were obtained from two plant breeding generations of *B.t.* Cry34/35Ab1 event DAS-59122-7, designated the BC2S1 generation and the T1S2 generation. The BC2S1 seed represents transformation into a Hi-II background followed by an outcross for one generation to inbred line PH09B, the resulting F1 was crossed and then backcrossed twice to inbred 581 to make a BC2. The BC2 generation was then self-crossed to generate a segregating population. The T1S2 generation seed consisted of the original Hi-II line containing event DAS-59122-7 (T0) crossed to elite inbred PH09B to give an F1 hybrid (T1), and then selfed twice to give T1S2 seed. Plants of both generations of event DAS-59122-7 were grown in growth chambers and leaf samples were obtained for genomic DNA extraction and analysis. Leaf samples of the unmodified control plants (Hi-II, PH09B, 581), which contain a genetic background representative of the transgenic plants, were also obtained for DNA extraction and analysis. Positive and negative plants within these two segregating generations were confirmed by testing with lateral flow test strips specific for the Cry34Ab1 protein and leaf painting with the herbicide glufosinate-ammonium to detect the expression of the PAT protein. For characterization of the inserted DNA in *B.t.* Cry34/35Ab1 event DAS-59122-7, genomic DNA samples of event DAS-59122-7 and unmodified control plants were digested with *Bsa* I, *Hin*d III, *Nco* I, *Sac* I and *Xho* I.

Following digestion with the restriction enzymes, the fragments produced were electrophoretically separated by size through an agarose gel and molecular weight markers were used to determine sufficient migration and separation of the fragments on the gel. The gel fragments were transferred to a nylon membrane via Southern blot transfer and were then detected as discrete bands via the use of DIG-labeled DNA probes (Table 4, Figure 2). Probes for the *cry*34Ab1 gene, the *cry*35Ab1 gene (combined 5' and 3' *cry*35Ab1 fragments homologous to this gene sequence), and the *pat* gene were used to detect the inserted transgenes. Probes for the ubiquitin promoter, wheat peroxidase promoter (combined 5' and 3' TA Perox Pro fragments homologous to this promoter), 35S promoter, ubiquitin intron, and pinII terminator were used to further characterize the insert. All DNA probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, from fragments generated by primers specific to gene elements from plasmid PHP17662 or plasmids containing equivalent elements.

The restriction enzymes *Bsa* I, *Sac* I, *Nco* I, *Hin*d III, and *Xho* I were selected for Southern blot characterization and are indicated on the PHP17662 T-DNA map in Figure 2. Genomic DNA samples from DAS-59122-7 plants and unmodified control DNA samples were digested with these enzymes and hybridized to various probes to characterize the insertion and provide information to generate a restriction enzyme map

of the region. Expected and observed fragment sizes with a particular digest and probe, based on the known restriction enzyme sites of the T-DNA fragment, are given in Tables 6 and 7. Two types of fragments were identified from these digests and hybridizations: internal fragments where known enzyme sites flank the probe region and are completely contained within the T-DNA region and border fragments where a known enzyme site is located at one end of the probe region and a second site is expected in the corn genome. Border fragments sizes vary by event because, in most cases, T-DNA integration sites are unique for each event. The border fragments provide a means to locate a restriction enzyme site relative to the integrated DNA and to evaluate the number of T-DNA insertions. Based on the Southern blot analyses completed in this study, it was hypothesized that a single copy of an intact T-DNA region from plasmid PHP17662 inserted into the corn genome of event DAS-59122-7 as detailed in the insert map (Figure 3). Results shown are primarily on the BC2S1 generation of event DAS-59122-7 plants, however identical results were obtained from analysis on T1S2 generation plants.

Table 6. Expected and observed hybridization fragments on Southern blots with gene element probes (DAS-59122-7).

DNA Probe	Restriction Enzymes	Figure	Expected Fragment Sizes (bp)[1]	Observed Fragment Size (bp)[2]
*cry*34Ab1	*Bsa* I	13	> 6372 (border)	~6400[3]
*cry*34Ab1	*Sac* I	13	> 3217 (border)	~3400
*cry*34Ab1	*Nco* I	13	1915	1915[4]
*cry*34Ab1	*Hin*d III	21	6963	6963[4]
*cry*34Ab1	*Xho* I	21	> 6568 (border)	~8000
*cry*35Ab1	*Bsa* I	14	> 6372 (border)	~6400[3]
cry35Ab1	*Sac* I	14	1941 1855 123[5]	1941[4] 1855[4]
*cry*35Ab1	*Nco* I	14	2607	2607[4]
*cry*35Ab1	*Hin*d III	22	6963	6963[4]
*cry*35Ab1	*Xho* I	22	> 6568 (border)	~8000
pat	*Bsa* I	15	> 1143 (border)	~2800
pat	*Sac* I	15	1855	1855[4]
pat	*Nco* I	15	> 1700 (border)	~3400
pat	*Hin*d III	23	6963	6963[4]
pat	*Xho* I	23	> 6568 (border)	~8000

[1]Expected fragment sizes are based on the T-DNA map of PHP17662 as shown in Figure 3.
[2]Observed fragments sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5–10% larger than their actual indicated molecular weight.
[3]The size of this fragment was estimated to be closer to 6400 bp, in contrast to the observed 6100 bp fragment on the Southern blots for two reasons: a complete fragment would not be less than the 6372 bp border expected and there is a discrepancy in the marker sizes as explained in footnote 2 of this table.
[4]Observed size of fragment or fragments is presumed to be the same as expected due to equivalent migration with bands in positive plasmid control PHP17662 lane.
[5]The expected 123 bp fragment was not detected, as fragments below approximately 700 bp were run off the gel during electrophoresis and were not transferred to the nylon membrane.

Table 7. Expected and observed hybridization fragments on Southern blots using promoter and intron element probes for event DAS-59122-7.

DNA probe	Restriction Enzymes	Figure	Expected Fragment Sizes (bp)[1]	Observed Fragment Size (bp)[2]
ubi pro	*Bsa* I	16	> 6372 (border)	~8000* ~6400[3]
ubi pro	*Sac* I	16	> 3217 (border)	~3600* ~3400
ubi pro	*Nco* I	16	> 1293 (border)	~6800 ~> 3600*
ubi pro	*Hin*d III	24	6963	6963[4]
ubi pro	*Xho* I	24	> 6568 (border) > 947 (border)	~8000 ~4900* ~1500 ~1400*
TA perox pro	*Bsa* I	17	> 6372 (border)	~6400[3] 3 bands ~1.48-2.8 kb*
TA perox pro	*Sac* I	17	> 3217 (border) 1941	~3400 1941[4] ~1500*
TA perox pro	*Nco* I	17	2607 1915	2607[4] 1915[4]
TA perox pro	*Hin*d III	25	6963	~> 8576* 6963[4]
35S pro	*Bsa* I	18	> 6372 (border)	~6400[3]
35S pro	*Sac* I	18	1855	1855[4]
35S pro	*Nco* I	18	> 1700 (border)	~3400
35S pro	*Hin*d III	26	6963	6963[4]
35S pro	*Xho* I	26	> 6568 (border)	~8000
ubi intron	*Bsa* I	19	> 6372 (border)	~8000* ~6400[3]
ubi intron	*Sac* I	19	> 3217 (border)	~3600* ~3400
ubi intron	*Nco* I	19	1915 > 1293 (border)	> 8600* 1 band ~2.8-3.6 kb* 1915[4] border not visible
ubi intron	*Hin*d III	27	6963	6963[4]
ubi intron	*Xho* I	27	> 6568 (border)	~8000 ~1400*
pinII term	*Bsa* I	20	> 6372 (border)	~6400[3]
pinII term	*Sac* I	20	> 3217 (border) 1941	~3400 1941[4]
pinII term	*Nco* I	20	2607 1915	2607[4] 1915[4]
pinII term	*Hin*d III	28	6963	6963[4]
pinII term	*Xho* I	28	> 6568 (border)	~8000

Note: An asterisk (*) after the observed fragment size indicates endogenous sequence hybridization.
[1]Expected fragment sizes are based on the T-DNA map of PHP17662 as shown in Figure 3.
[2]Observed fragments sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5-10% larger than their actual indicated molecular weight.
[3]The size of this fragment was estimated to be closer to 6400 bp, in contrast to the observed 6100 bp fragment on the Southern blots for two reasons: a complete fragment would not be less than the 6372 bp border expected and there is a discrepancy in the marker sizes as explained in footnote 2 of this table.
[4]Observed size of fragment or fragments is presumed to be the same as expected due to equivalent migration with bands in positive plasmid control PHP17662 lane.

Bsa I analysis of event DAS-59122-7 confirmed a single T-DNA insertion in the corn genome as all probes hybridized to a single fragment (Tables 6 and 7). The *cry*34Ab1 (Figures 13), *cry*35Ab1 (Figure 14), ubiquitin promoter (Figure 16), wheat peroxidase promoter (Figure 17), 35S promoter (Figure 18), ubiquitin intron (Figure 19), and the pin II terminator (Figure 20) probes hybridized to a single *Bsa* I fragment of approximately 6400 bp in event DAS-59122-7 (Tables 6 and 7), indicating a restriction enzyme site just outside the T-DNA Right Border region (Figure 15). Likewise, the *pat* probe (Figure 3) hybridized to a single *Bsa* I fragment of approximately 2800 bp (Table 6) indicating that the site was located approximately 1.7 kb outside the T-DNA Left Border region in the corn genome (Figure 3).

In addition, both *Sac* I and *Nco* I analysis further confirmed a single T-DNA insertion in DAS-59122-7 and placed these enzyme sites in genomic regions adjacent to the insertion (Figure 3). In the *Sac* I analysis, the *cry*34Ab1 (Figure 13, Table 6), ubiquitin promoter (Figure 16, Tables 7), wheat peroxidase promoter (Figure 17, Table 7), ubiquitin intron (Figure 19, Table 7), and pin II terminator (Figure 20, Table 7) probes hybridized to a single fragment of approximately 3400 bp in DAS-59122-7, placing a *Sac* I site just outside the T-DNA Right Border region (Figure 3). The ubiquitin promoter probe hybridized to a single fragment of approximately 6800 bp in the *Nco* I analysis (Figure 16, Table 7), placing the site approximately 5.5 kb outside the T-DNA Right Border region of the event (Figure 3). Both the *pat* probe (Figure 15, Table 6) and the 35S promoter probe (Figure 18, Table 7) hybridized to a single fragment of approximately 3400 bp, indicating an *Nco* I site 1.7 kb outside of the Left Border T-DNA region (Figure 3).

The presence of hybridizing internal fragments in event DAS-59122-7 was confirmed by analysis with *Sac* I, *Nco* I, and *Hin*d III. In the *Sac* I analysis, the *cry*35Ab1 probe hybridized to two fragments at approximately 1.9 kb in the event (Figure 14, Table 6) which correlate with the expected fragments of 1855 bp and 1941 bp. The *pat* probe hybridized to the 1855 bp fragment alone (Figure 15, Table 6). These expected fragments were also observed from hybridizations with the wheat peroxidase promoter probe (1941 bp, Figure 17, Table 7), with the 35S promoter probe (1855 bp, Figure 18, Table 7), and with the pin II terminator probe (1941 bp, Figure 20, Table 7). In the *Nco* I analysis, two major internal fragments, 1915 bp and 2607 bp, were visible with hybridizations to several of the probes. The *cry*34Ab1 probe (Figure 13, Table 6) and the ubiquitin intron probe (Figure 19, Table 7) hybridized to the expected 1915 bp fragment and the *cry*35Ab1 hybridized to the expected 2607 bp fragment (Figure 14, Table 6) in event DAS-59122-7. In addition, the wheat peroxidase promoter probe hybridized to both of these expected fragments (1915 bp and 2607 bp, Figure 17, Table 7), as did the pin II terminator probe (Figure 20, Table 7).

*Hin*d III was used to evaluate the presence of an intact insertion, as sites for this enzyme flank the *cry*34Ab1, *cry*35Ab1, and *pat* gene elements in the T-DNA (Figure 2, *Hin*d III sites). In event DAS-59122-7, the *cry*34Ab1 (Figure 21), *cry*35Ab1 (Figure 22), *pat*

(Figure 23), ubiquitin promoter (Figure 24), wheat peroxidase promoter (Figure 25), 35S promoter (Figure 26), ubiquitin intron (Figure 27), and pinII terminator (Figure 28) probes hybridized to a fragment that migrated with the expected PHP17662 fragment of 6963 bp (Tables 6 and 7). Based on these results, and the absence of other hybridizing fragments, it was concluded that the T-DNA inserted as an intact copy.

Similar to *Bsa* I, *Xho* I also confirmed a single T-DNA insertion in event DAS-59122-7 (Tables 6 and 7). The *cry*34Ab1 (Figure 21), *cry*35Ab1 (Figure 22), *pat* (Figure 23), 35S promoter (Figure 26), ubiquitin intron (Figure 27), and pinII terminator (Figure 28) probes hybridized to a single fragment of approximately 8000 bp (Tables 6 and 7), indicating an *Xho* I site between 800 bp and 1.4 kb outside the T-DNA Left Border region (Figure 3). In addition, the ubiquitin promoter probe (Figure 24) hybridized to a fragment approximately 1.5 kb in size (Table 7) and placed an *Xho* I site approximately 600 bp outside the T-DNA Right Border region of the insertion (Figure 3).

The Southern hybridization results combined from the two generation study (Locke and Igo, 2003; Appendix 2, Section 6) and the detailed characterization study (Locke *et al.*, 2003; Appendix 2, Section 7) indicated that a single, intact T-DNA inserted in the genomic DNA of event DAS-59122-7 and sites for *Bsa* I, *Sac* I, *Nco* I, and *Xho* I were placed relative to the Right and Left Border regions of the T-DNA insertion (Figure 3). In addition, the results did not indicate that rearrangements of the T-DNA had occurred, as all expected internal restriction enzyme sites appeared to be intact and produced hybridizing fragments of expected size (Tables 5, 6, and 7).

Figure 13. Southern blot analysis of DAS-59122-7; *cry*34Ab1 probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I, and *Nco* I and probed with the *cry*34Ab1 gene probe. (T42 and T45 refer to plant ID 03-14C-T42 and 03-14C-T45, respectively.) Based on the stained gel of this blot, estimated amounts of genomic DNA loaded per lane are indicated below to explain differences in observed band intensities. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	µg	Digest
1	DIG VII		–
2	1 Copy PHP17662 + Hi-II	2-3	Bsa I
3	Hi-II (control)	2-3	Bsa I
4	PH09B (control)	5	Bsa I
5	DAS-59122-7 T42 (BC2S1)	4	Bsa I
6	DAS-59122-7 T45 (BC2S1)	5	Bsa I
7	DIG VII		–
8	1 Copy PHP17662 + Hi-II	2-3	Sac I
9	Hi-II (control)	5	Sac I
10	PH09B (control)	5	Sac I

Lane	Sample	µg	Digest
11	DAS-59122-7 T42 (BC2S1)	5	Sac I
12	DAS-59122-7 T45 (BC2S1)	2-3	Sac I
13	DIG VII		–
14	1 Copy PHP17662 + Hi-II	4	Nco I
15	Hi-II (control)	5	Nco I
16	PH09B (control)	2-3	Nco I
17	DAS-59122-7 T42 (BC2S1)	4	Nco I
18	DAS-59122-7 T45 (BC2S1)	1	Nco I
19	DIG VII		–

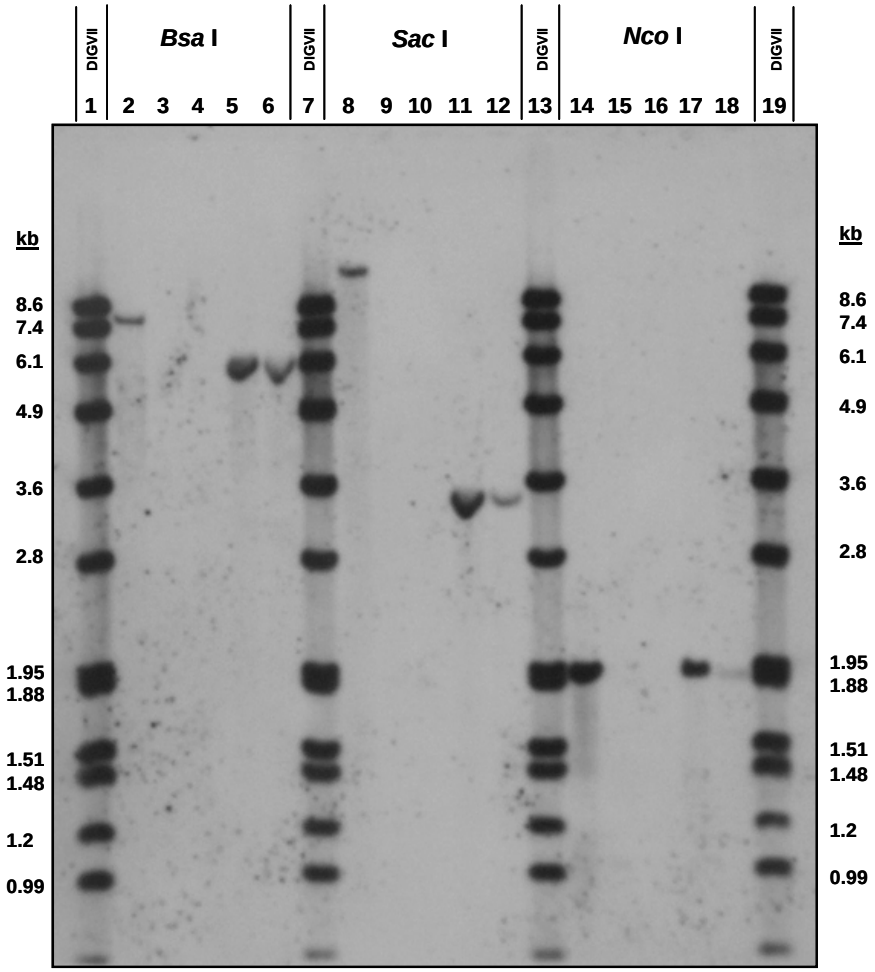


Figure 14. Southern blot analysis of DAS-59122-7; *cry*35Ab1 probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the *cry*35Ab1 gene probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 g of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 g of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Two fragments were observed in lanes 8, 11, and 12 on film exposures; however, the lower band is faintly visible in this exposure presented here.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Bsa I
3	Hi-II (control)	Bsa I
4	PH09B (control)	Bsa I
5	DAS-59122-7 T49 (BC2S1)	Bsa I
6	DAS-59122-7 T50 (BC2S1)	Bsa I
7	DIG VII	–
8	1 Copy PHP17662 + Hi-II	Sac I
9	Hi-II (control)	Sac I
10	PH09B (control)	Sac I

Lane	Sample	Digest
11	DAS-59122-7 T49 (BC2S1)	Sac I
12	DAS-59122-7 T50 (BC2S1)	Sac I
13	DIG VII	–
14	1 Copy PHP17662 + Hi-II	Nco I
15	Hi-II (control)	Nco I
16	PH09B (control)	Nco I
17	DAS-59122-7 T49 (BC2S1)	Nco I
18	DAS-59122-7 T50 (BC2S1)	Nco I
19	DIG VII	–

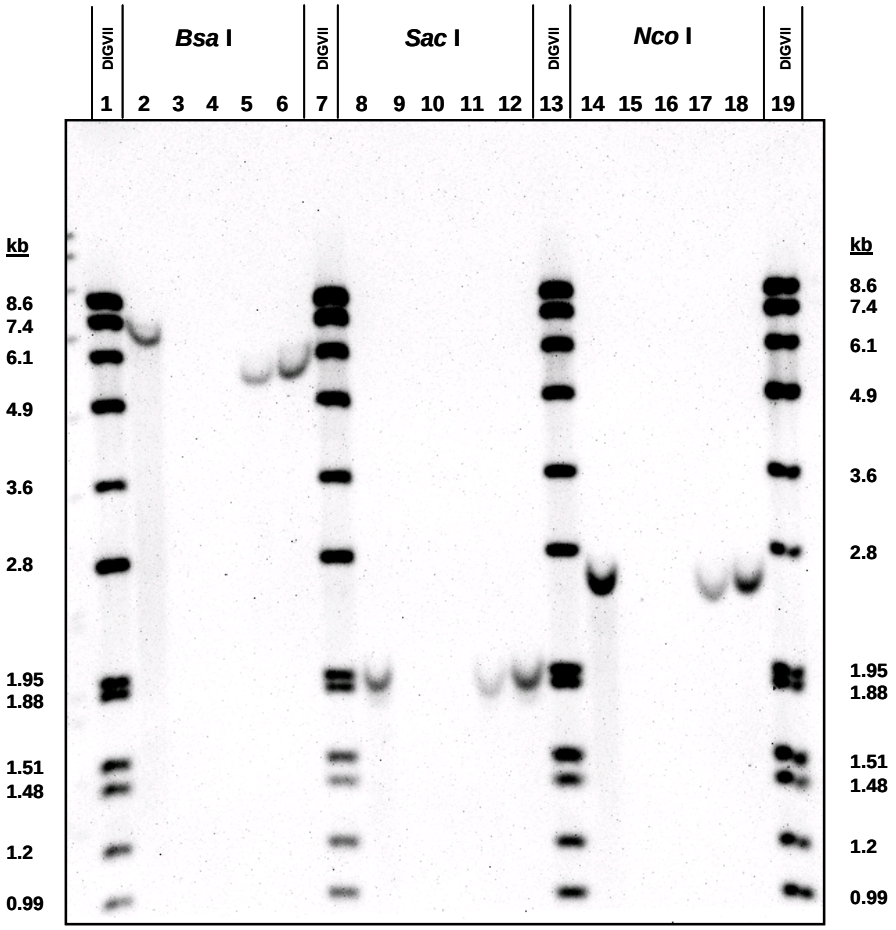


Figure 15. Southern blot analysis of DAS-59122-7; *pat* probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the *pat* gene probe. (T42 and T45 refer to plant ID 03-14C-T42 and 03-14C-T45, respectively.) Based on the stained gel of this blot, estimated amounts of genomic DNA loaded per lane are indicated below to explain differences in observed band intensities. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	µg	Digest
1	DIG VII		–
2	1 Copy PHP17662 + Hi-II	2-3	Bsa I
3	Hi-II (control)	2-3	Bsa I
4	PH09B (control)	5	Bsa I
5	DAS-59122-7 T42 (BC2S1)	4	Bsa I
6	DAS-59122-7 T45 (BC2S1)	5	Bsa I
7	DIG VII		–
8	1 Copy PHP17662 + Hi-II	2-3	Sac I
9	Hi-II (control)	5	Sac I
10	PH09B (control)	5	Sac I

Lane	Sample	µg	Digest
11	DAS-59122-7 T42 (BC2S1)	5	Sac I
12	DAS-59122-7 T45 (BC2S1)	2-3	Sac I
13	DIG VII		–
14	1 Copy PHP17662 + Hi-II	4	Nco I
15	Hi-II (control)	5	Nco I
16	PH09B (control)	2-3	Nco I
17	DAS-59122-7 T42 (BC2S1)	4	Nco I
18	DAS-59122-7 T45 (BC2S1)	1	Nco I
19	DIG VII		–

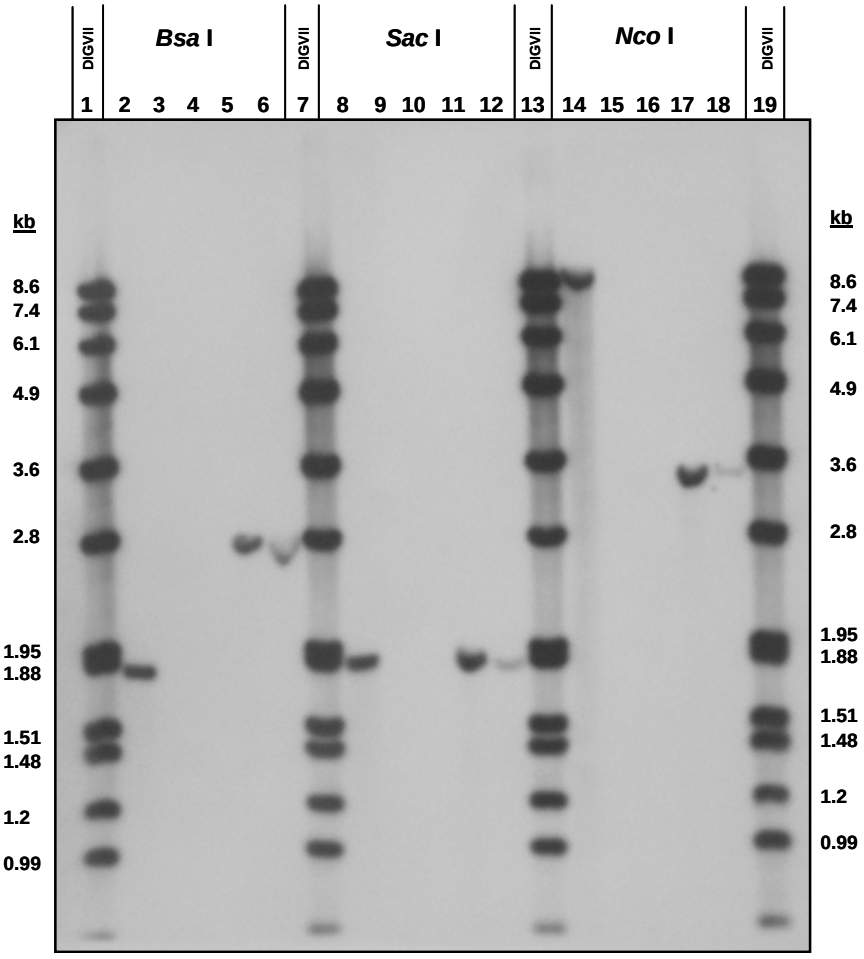


Figure 16. Southern blot analysis of DAS-59122-7; ubiquitin promoter probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the ubiquitin promoter probe. (T42 and T45 refer to plant ID 03-14C-T42 and 03-14C-T45, respectively.) Based on the stained gel of this blot, estimated amounts of genomic DNA loaded per lane are indicated below to explain differences in observed band intensities. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.
Note: Probe hybridizes to endogenous sequences in the corn genome (Table 7).
Lane assignments:

Lane	Sample	µg	Digest
1	DIG VII		–
2	1 Copy PHP17662 + Hi-II	2-3	Bsa I
3	Hi-II (control)	2-3	Bsa I
4	PH09B (control)	5	Bsa I
5	DAS-59122-7 T42 (BC2S1)	4	Bsa I
6	DAS-59122-7 T45 (BC2S1)	5	Bsa I
7	DIG VII		–
8	1 Copy PHP17662 + Hi-II	2-3	Sac I
9	Hi-II (control)	5	Sac I
10	PH09B (control)	5	Sac I

Lane	Sample	µg	Digest
11	DAS-59122-7 T42 (BC2S1)	5	Sac I
12	DAS-59122-7 T45 (BC2S1)	2-3	Sac I
13	DIG VII		–
14	1 Copy PHP17662 + Hi-II	4	Nco I
15	Hi-II (control)	5	Nco I
16	PH09B (control)	2-3	Nco I
17	DAS-59122-7 T42 (BC2S1)	4	Nco I
18	DAS-59122-7 T45 (BC2S1)	1	Nco I
19	DIG VII		–

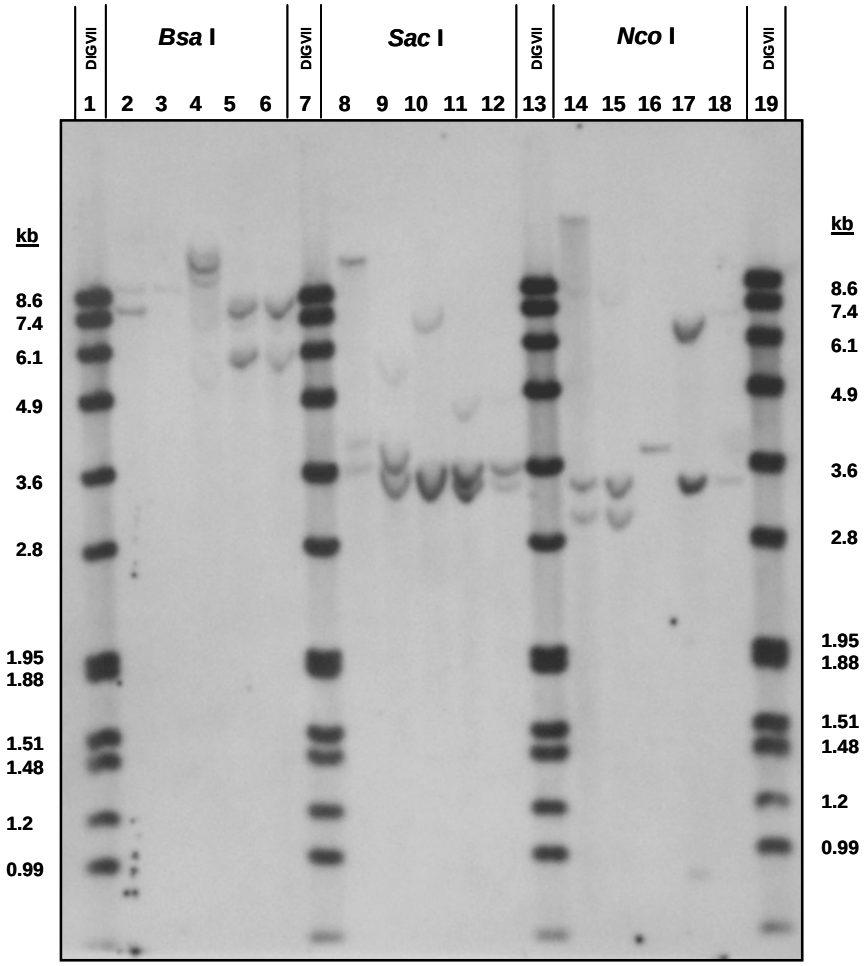


Figure 17. Southern blot analysis of DAS-59122-7; TA perox promoter probe; *Bsa* I, *Sac* I and *Nco* I digests.

DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the wheat peroxidase promoter probe. (T42 and T45 refer to plant ID 03-14C-T42 and 03-14C-T45, respectively.) Based on the stained gel of this blot, estimated amounts of genomic DNA loaded per lane are indicated below to explain differences in observed band intensities. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Probe hybridizes to endogenous sequences in the corn genome (Table 7).

Lane assignments:

Lane	Sample	µg	Digest
1	DIG VII		–
2	1 Copy PHP17662 + Hi-II	2-3	Bsa I
3	Hi-II (control)	2-3	Bsa I
4	PH09B (control)	5	Bsa I
5	DAS-59122-7 T42 (BC2S1)	4	Bsa I
6	DAS-59122-7 T45 (BC2S1)	5	Bsa I
7	DIG VII		–
8	1 Copy PHP17662 + Hi-II	2-3	Sac I
9	Hi-II (control)	5	Sac I
10	PH09B (control)	5	Sac I

Lane	Sample	µg	Digest
11	DAS-59122-7 T42 (BC2S1)	5	Sac I
12	DAS-59122-7 T45 (BC2S1)	2-3	Sac I
13	DIG VII		–
14	1 Copy PHP17662 + Hi-II	4	Nco I
15	Hi-II (control)	5	Nco I
16	PH09B (control)	2-3	Nco I
17	DAS-59122-7 T42 (BC2S1)	4	Nco I
18	DAS-59122-7 T45 (BC2S1)	1	Nco I
19	DIG VII		–

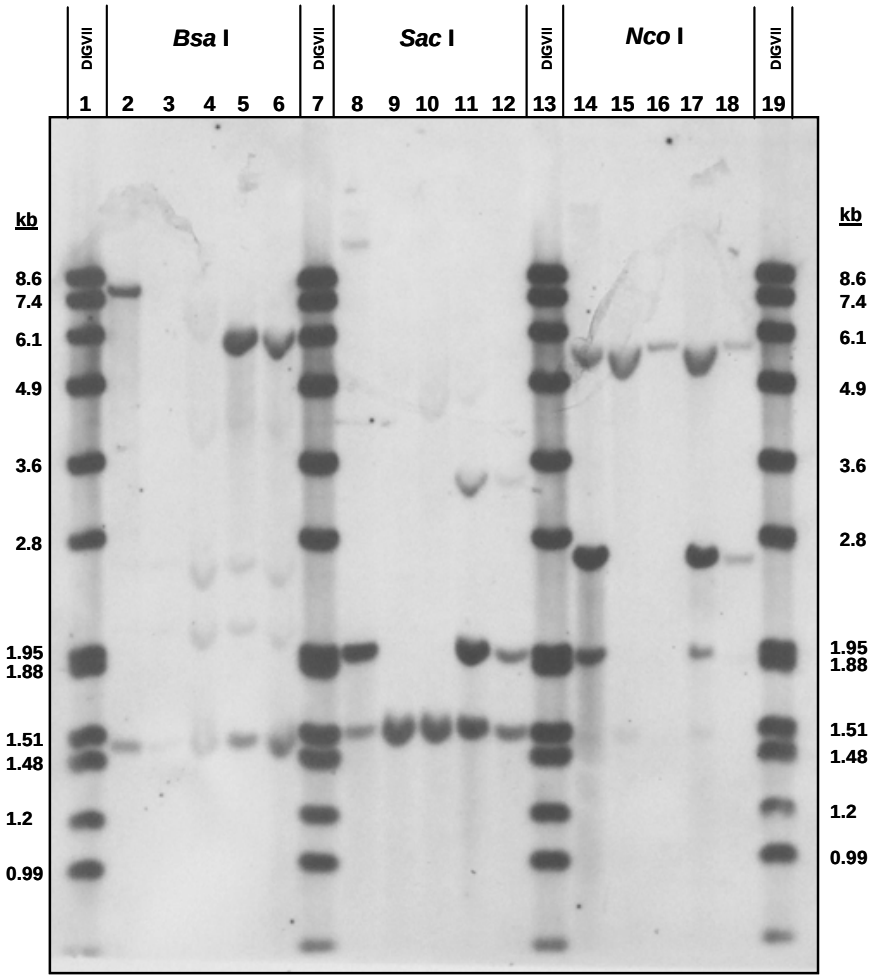


Figure 18. Southern blot analysis of DAS-59122-7; 35S promoter probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the 35S promoter probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Significant background splotches are visible around the 0.99-1.2 kb marker of lanes 2, 3, and 15 and around the 6.1-7.4 kb marker of lanes 14 and 15.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Bsa I
3	Hi-II (control)	Bsa I
4	PH09B (control)	Bsa I
5	DAS-59122-7 T49 (BC2S1)	Bsa I
6	DAS-59122-7 T50 (BC2S1)	Bsa I
7	DIG VII	–
8	1 Copy PHP17662 + Hi-II	Sac I
9	Hi-II (control)	Sac I
10	PH09B (control)	Sac I

Lane	Sample	Digest
11	DAS-59122-7 T49 (BC2S1)	Sac I
12	DAS-59122-7 T50 (BC2S1)	Sac I
13	DIG VII	–
14	1 Copy PHP17662 + Hi-II	Nco I
15	Hi-II (control)	Nco I
16	PH09B (control)	Nco I
17	DAS-59122-7 T49 (BC2S1)	Nco I
18	DAS-59122-7 T50 (BC2S1)	Nco I
19	DIG VII	–

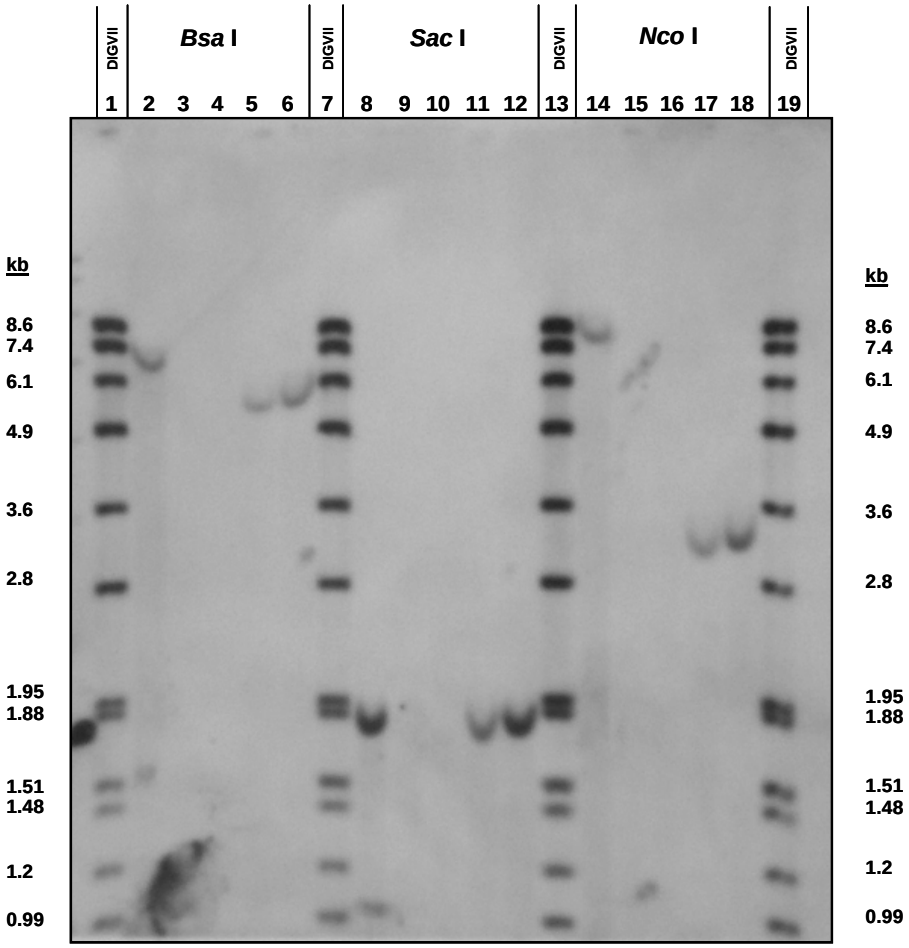


Figure 19. Southern blot analysis of DAS-59122-7; ubiquitin intron probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I and *Nco* I and probed with the ubiquitin intron probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Probe hybridizes to endogenous sequences in the corn genome (Table 7).

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Bsa I
3	Hi-II (control)	Bsa I
4	PH09B (control)	Bsa I
5	DAS-59122-7 T49 (BC2S1)	Bsa I
6	DAS-59122-7 T50 (BC2S1)	Bsa I
7	DIG VII	–
8	1 Copy PHP17662 + Hi-II	Sac I
9	Hi-II (control)	Sac I
10	PH09B (control)	Sac I

Lane	Sample	Digest
11	DAS-59122-7 T49 (BC2S1)	Sac I
12	DAS-59122-7 T50 (BC2S1)	Sac I
13	DIG VII	–
14	1 Copy PHP17662 + Hi-II	Nco I
15	Hi-II (control)	Nco I
16	PH09B (control)	Nco I
17	DAS-59122-7 T49 (BC2S1)	Nco I
18	DAS-59122-7 T50 (BC2S1)	Nco I
19	DIG VII	–

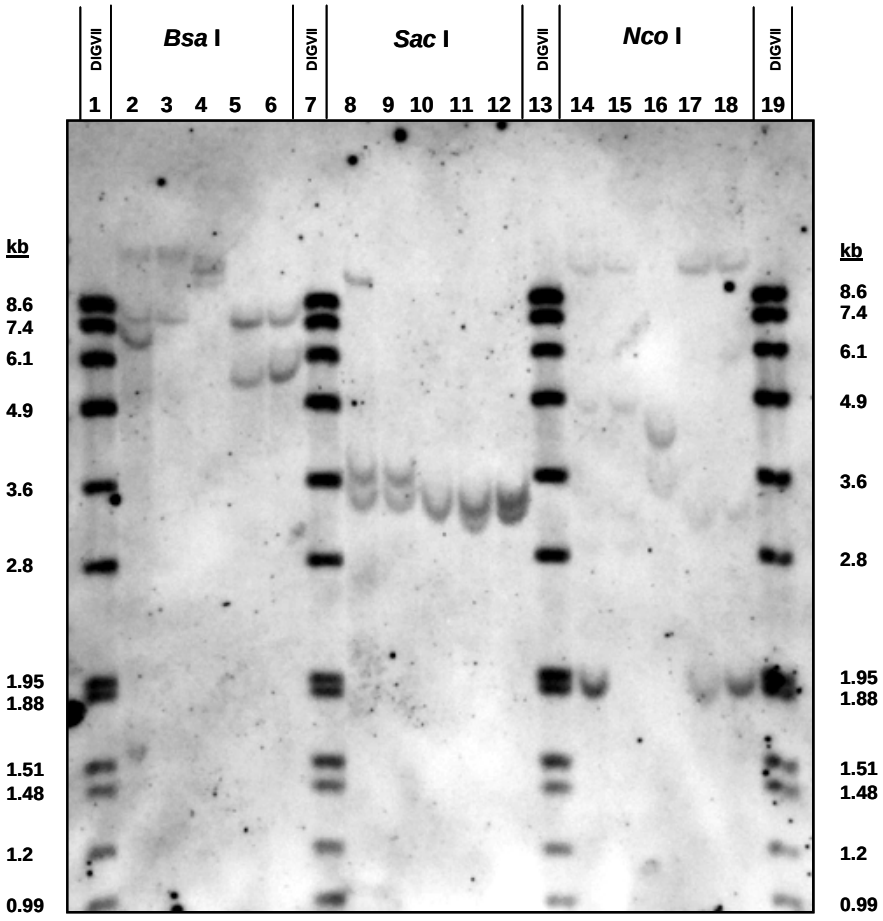


Figure 20. Southern blot analysis of DAS-59122-7; pin II terminator probe; *Bsa* I, *Sac* I and *Nco* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Bsa* I, *Sac* I, and *Nco* I and probed with the pin II terminator probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Significant background splotches visible around the 0.99-1.2 kb marker in lanes 2 and 3.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Bsa I
3	Hi-II (control)	Bsa I
4	PH09B (control)	Bsa I
5	DAS-59122-7 T49 (BC2S1)	Bsa I
6	DAS-59122-7 T50 (BC2S1)	Bsa I
7	DIG VII	–
8	1 Copy PHP17662 + Hi-II	Sac I
9	Hi-II (control)	Sac I
10	PH09B (control)	Sac I

Lane	Sample	Digest
11	DAS-59122-7 T49 (BC2S1)	Sac I
12	DAS-59122-7 T50 (BC2S1)	Sac I
13	DIG VII	–
14	1 Copy PHP17662 + Hi-II	Nco I
15	Hi-II (control)	Nco I
16	PH09B (control)	Nco I
17	DAS-59122-7 T49 (BC2S1)	Nco I
18	DAS-59122-7 T50 (BC2S1)	Nco I
19	DIG VII	–

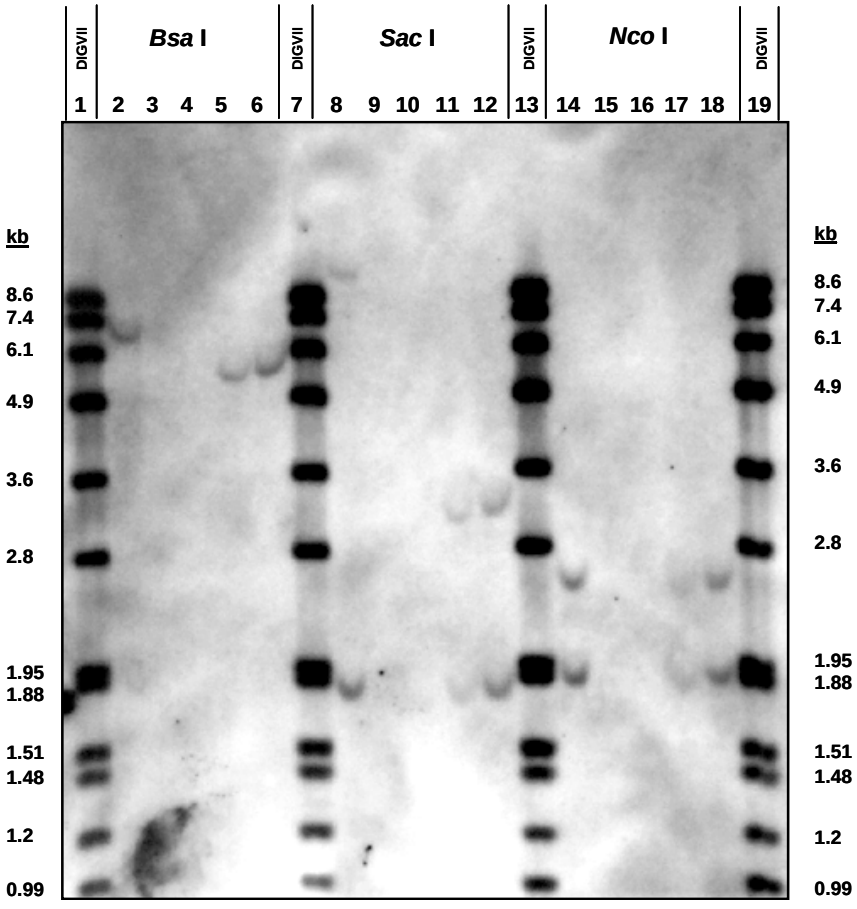


Figure 21. Southern blot analysis of DAS-59122-7; *cry*34Ab1 probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the *cry*34Ab1 gene probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–

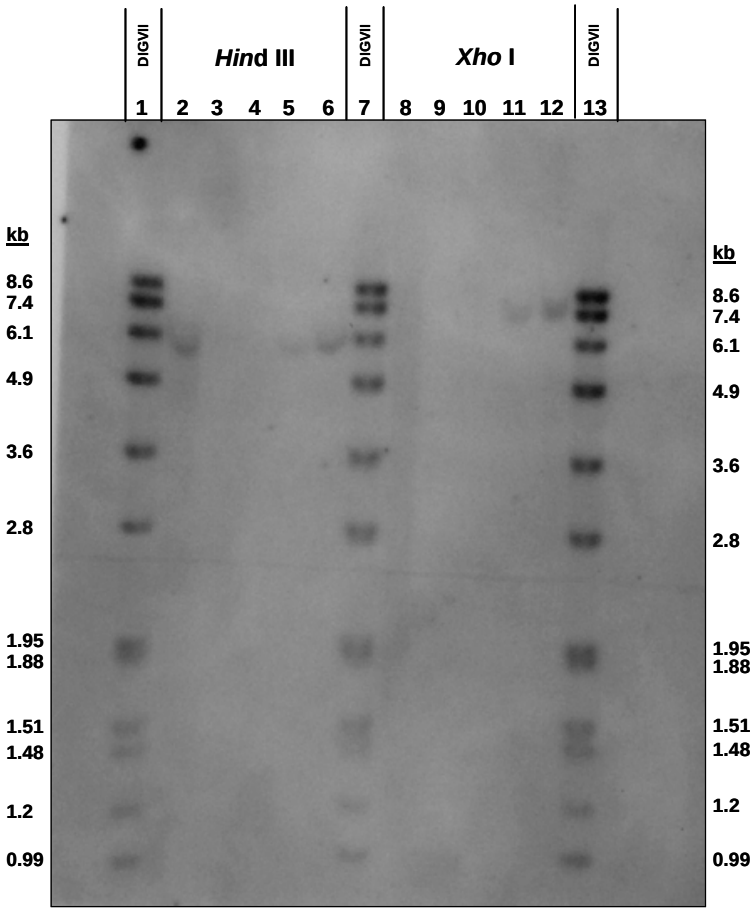


Figure 22. Southern blot analysis of DAS-59122-7; *cry*35Ab1 probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the *cry*35Ab1 gene probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 Copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–

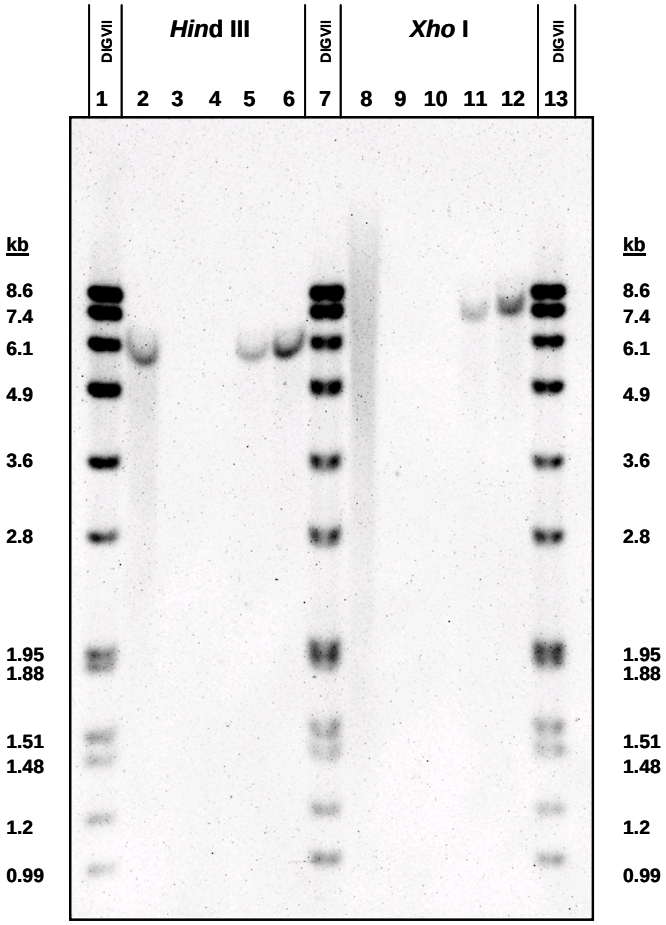


Figure 23. Southern blot analysis of DAS-59122-7; *pat* probe; *Hin*d III and *Xho* I digests.
DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the *pat* gene probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–

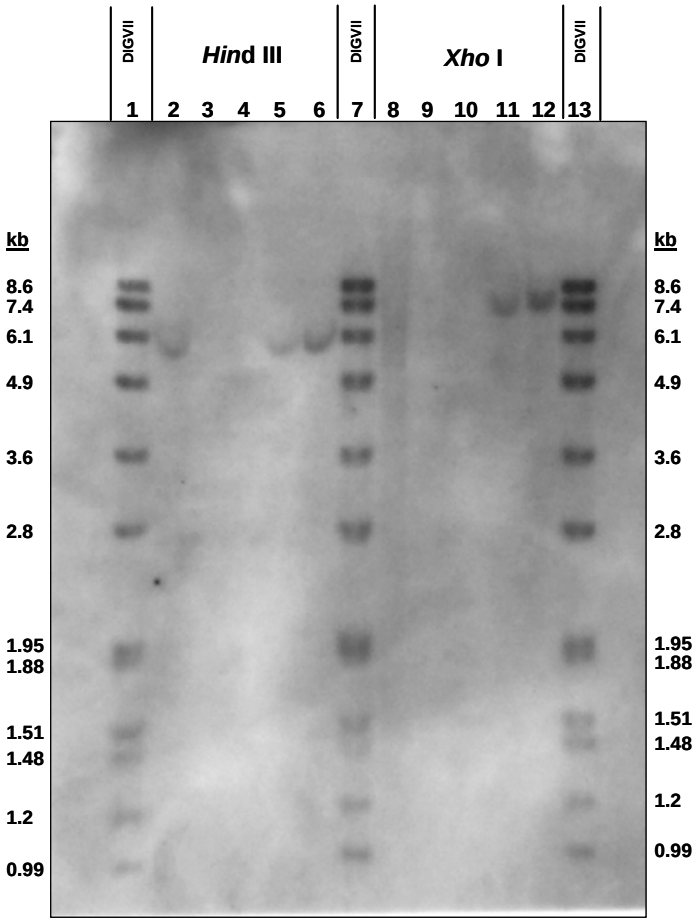


Figure 24. Southern blot analysis of DAS-59122-7; ubiquitin promoter probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (T1S2) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the ubiquitin promoter probe. (T2 and T10 refer to plant ID 02-122C-2 and 02-122C-10, respectively.) Approximately 7 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Probe hybridizes to endogenous sequences in the corn genome (Table 7).

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T2 (T1S2)	Hind III
6	DAS-59122-7 T10 (T1S2)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T2 (T1S2)	Xho I
12	DAS-59122-7 T10 (T1S2)	Xho I
13	DIG VII	–

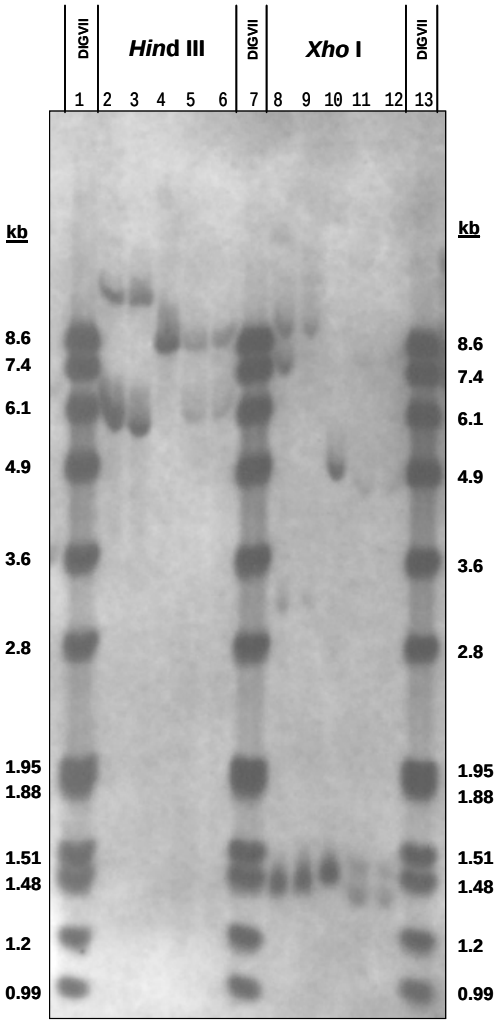


Figure 25. Southern blot analysis of DAS-59122-7; TA perox promoter probe; *Hin*d III digest. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B, 581 and Hi-II unmodified corn was digested with *Hin*d III and probed with the wheat peroxidase promoter probe. (T3 and T1 refer to plant ID 03-14C-T3 and 03-14C-T1, respectively.) Approximately 7□μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 μg of unmodified PH09B DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Probe hybridizes to endogenous sequences in the corn genome (Table 7).

Lane assignments:

Lane	Sample	Digest
1	1 copy PHP17662 + PH09B	–
2	PH09B (Control)	Hind III
3	Hi-II (Control)	Hind III
4	581 (Control)	Hind III
5	DAS-59122-7 T3 (BC2S1)	Hind III
6	DAS-59122-7 T1 (BC2S1)	Hind III
7	DIG VII	–

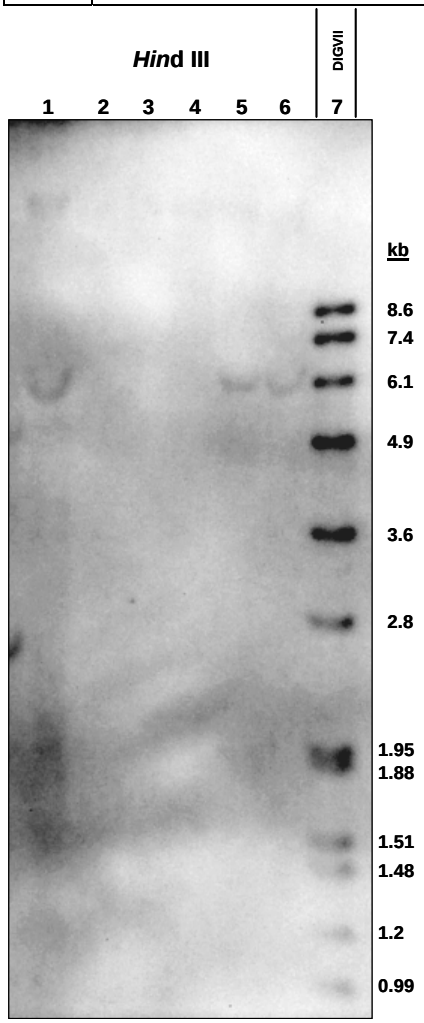


Figure 26. Southern blot analysis of DAS-59122-7; 35S promoter probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the 35S promoter probe. (T49 and T50 refer to plant 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 Copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–

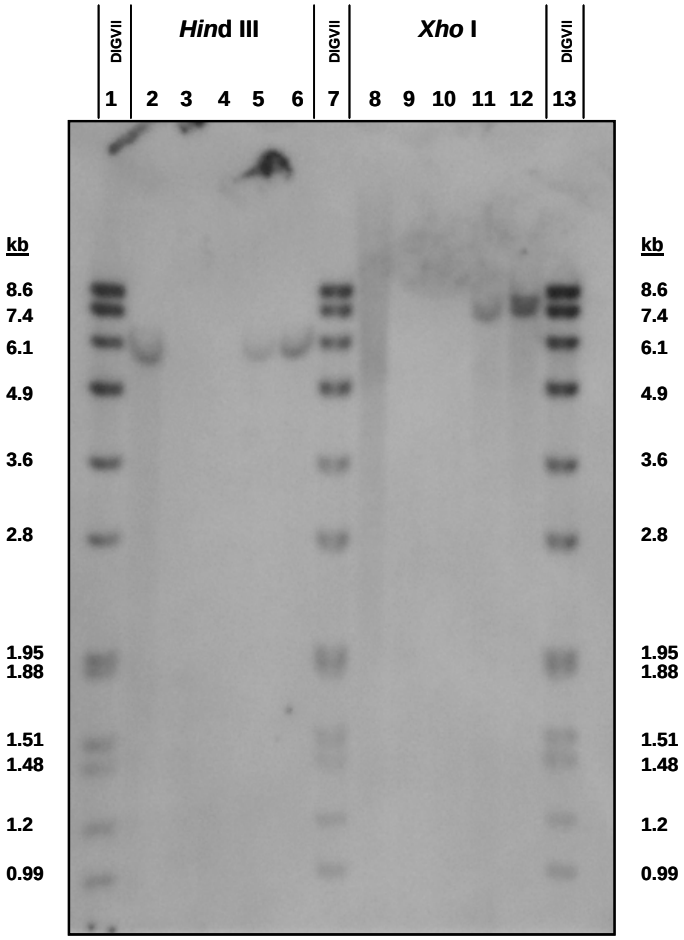


Figure 27. Southern blot analysis of DAS-59122-7; ubiquitin intron probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the ubiquitin intron probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Probe hybridizes to endogenous sequences in the corn genome (Table 7). Significant background spot visible in lane 9, just above the 1.95 kb marker.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 Copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–

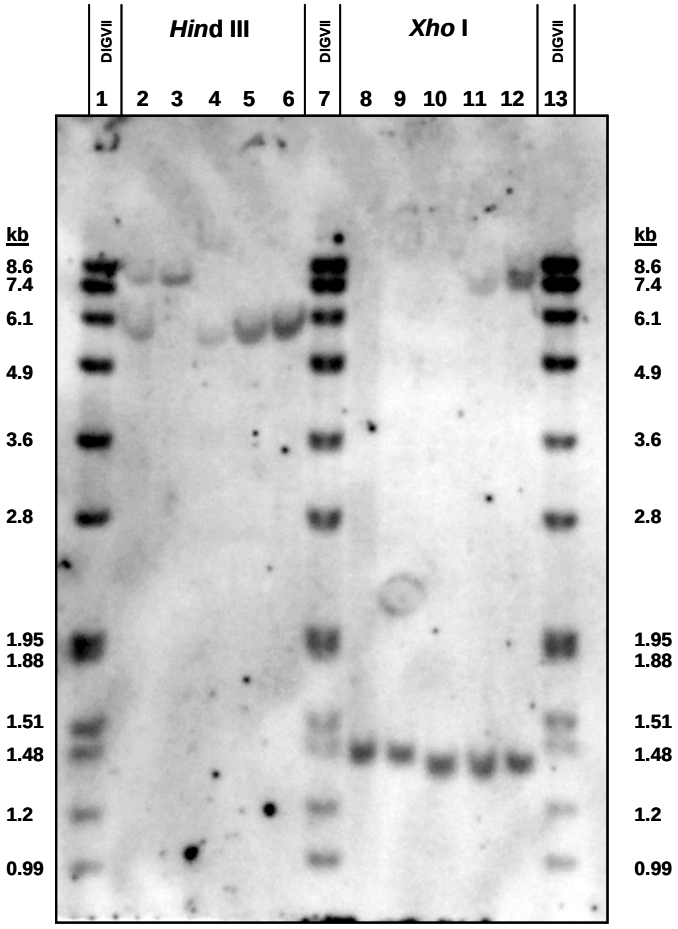
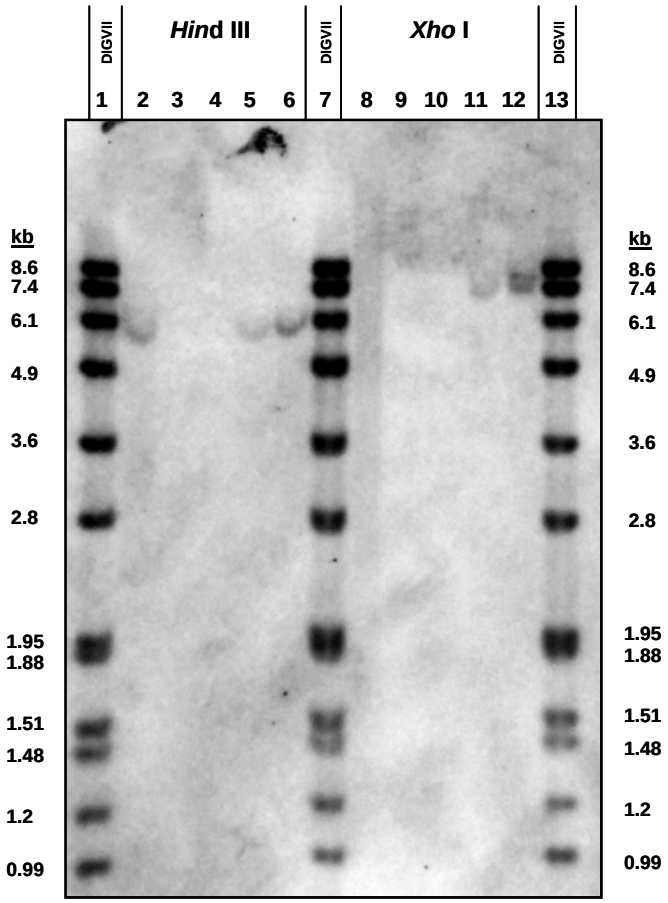


Figure 28. Southern blot analysis of DAS-59122-7; pin II terminator probe; *Hin*d III and *Xho* I digests. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B and Hi-II unmodified corn was digested with *Hin*d III and *Xho* I and probed with the pin II terminator probe. (T49 and T50 refer to plant ID 03-14C-T49 and 03-14C-T50, respectively.) Approximately 5 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 5 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane assignments:

Lane	Sample	Digest
1	DIG VII	–
2	1 Copy PHP17662 + Hi-II	Hind III
3	Hi-II (control)	Hind III
4	PH09B (control)	Hind III
5	DAS-59122-7 T49 (BC2S1)	Hind III
6	DAS-59122-7 T50 (BC2S1)	Hind III
7	DIG VII	–

Lane	Sample	Digest
8	1 Copy PHP17662 + Hi-II	Xho I
9	Hi-II (control)	Xho I
10	PH09B (control)	Xho I
11	DAS-59122-7 T49 (BC2S1)	Xho I
12	DAS-59122-7 T50 (BC2S1)	Xho I
13	DIG VII	–



Absence of Tetracylcine and Spectinomycin Resistance Genes and Absence of Regions Immediately Outside of the T-DNA Borders

Southern blot analysis of DNA extracted from corn leaf tissue was used to confirm the absence of tetracycline (*tet*), spectinomycin (*spc*), and *vir*G genes and two other regions

that are present outside of the T-DNA borders of the transformation vector (Locke and Igo, 2003; Appendix 2, Section 6).

DNA samples from transgenic corn event DAS-59122-7, T1S1 and BC1 generations, and unmodified controls were digested with *Hin*d III, *Sac* I, and *Xho* I and probed with one or more of the five backbone probes; *spc*, *tet*, *vir*G, LB Backbone, and RB Backbone, to confirm the absence of the spectinomycin and tetracycline resistance genes, the *vir*G gene, and the absence of regions immediately outside of the T-DNA that were contained on the vector backbone of plasmid PHP17662 (Table 3). Positive controls consisting of unmodified control corn DNA spiked with plasmid PHP17662 DNA at approximately one and three gene copy equivalents were digested with *Hin*d III, *Sac* I, and *Xho* I to confirm successful probe hybridization. Negative controls consisted of DNA from unmodified corn samples.

No hybridization signals were evident in any of the transgenic corn event DAS-59122-7 samples, null segregants of the event, or unmodified controls when hybridized to the *spc*, *tet*, *vir*G, LB Backbone, and RB Backbone probes (Figure 29 – Figure 36 and Table 8). The positive control lanes contained the expected hybridizing bands demonstrating that the probes were capable of hybridizing to any homologous DNA fragments if present in the samples. The data indated that as expected the insertion in transgenic corn event DAS-59122-7 did not include sequence outside of the T-DNA borders from plasmid PHP17662.

Table 8. Predicted and observed hybridizing bands on Southern blots of transgenic corn event DAS-59122-7 probed with the *spc*, *tet*, *vir*G, RB backbone, and LB backbone probes.

Probe	Restriction Enzyme	Figure	Predicted Fragment Size from T-DNA[1] (bp)	Predicted Fragment Size from Plasmid PHP17662[1] (bp)	Observed Fragment Size (bp)
spc	*Hin*d III	29	None	25076	None
spc	*Sac* I	30	None	25114	None
tet	*Hin*d III	31	None	25076	None
tet	*Sac* I	32	None	25114	None
*vir*G	*Sac* I	33	None	6980	None
LB Backbone	*Sac* I	34	None	25114	None
LB Backbone	*Xho* I	35	None	31309	None
RB Backbone	*Hin*d III	36	None	3605	None

[1]Predicted fragment sizes are based on the plasmid map and the T-DNA map of PHP17662 as shown in Figures 1 and 2, respectively.

Figure 29. Southern blot analysis of DAS-59122-7; Hind III digest, spc probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the spc gene probe. Approximately 7µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. Note: The dark streaks at approximately 1.7 kb that extend across lanes 1 and 2 and lanes 3, 4, and 5 are due to a scratch on the membrane surface and are not associated with probe hybridization to DNA in the lanes.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7-T52 (s) T1S1	Hind III
6	DAS-59122-7-T46 T1S1	Hind III
7	DAS-59122-7-T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

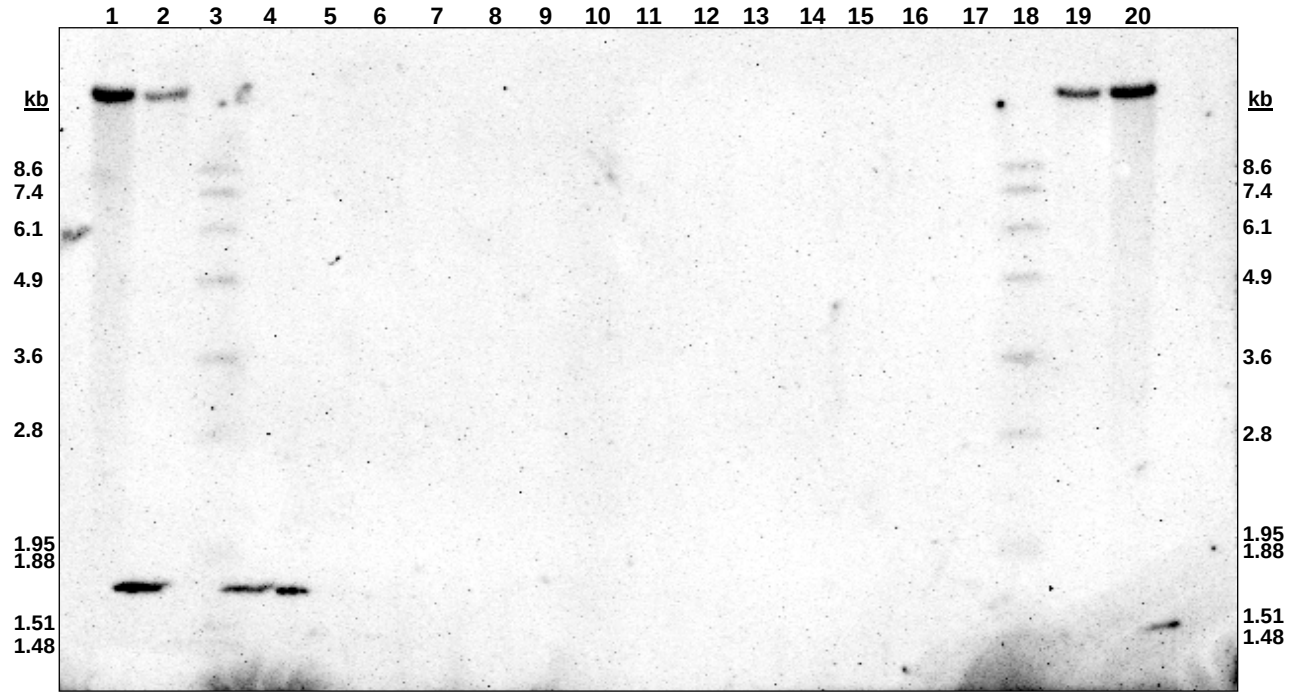


Figure 30. Southern blot analysis of DAS-59122-7; Sac I digest, spc probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the spc gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

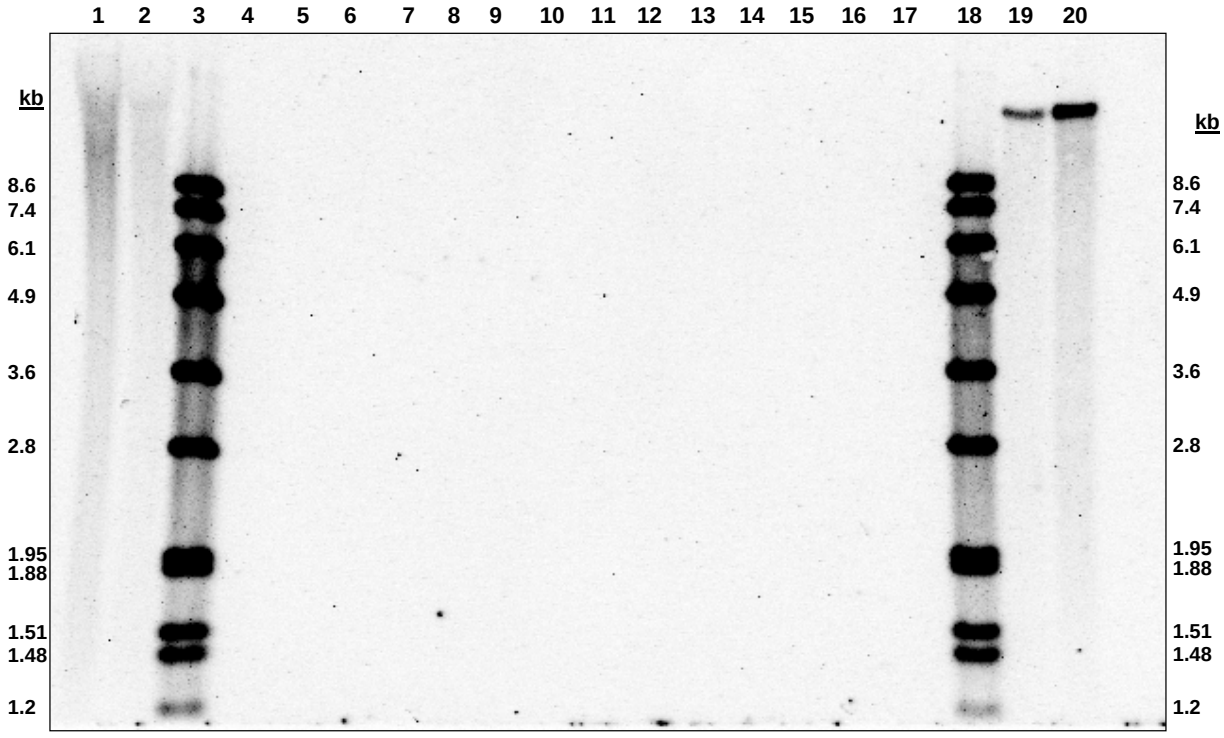


Figure 31. Southern blot analysis of DAS-59122-7; Hind III digest, tet probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the tet gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7-T52 (s) T1S1	Hind III
6	DAS-59122-7-T46 T1S1	Hind III
7	DAS-59122-7-T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

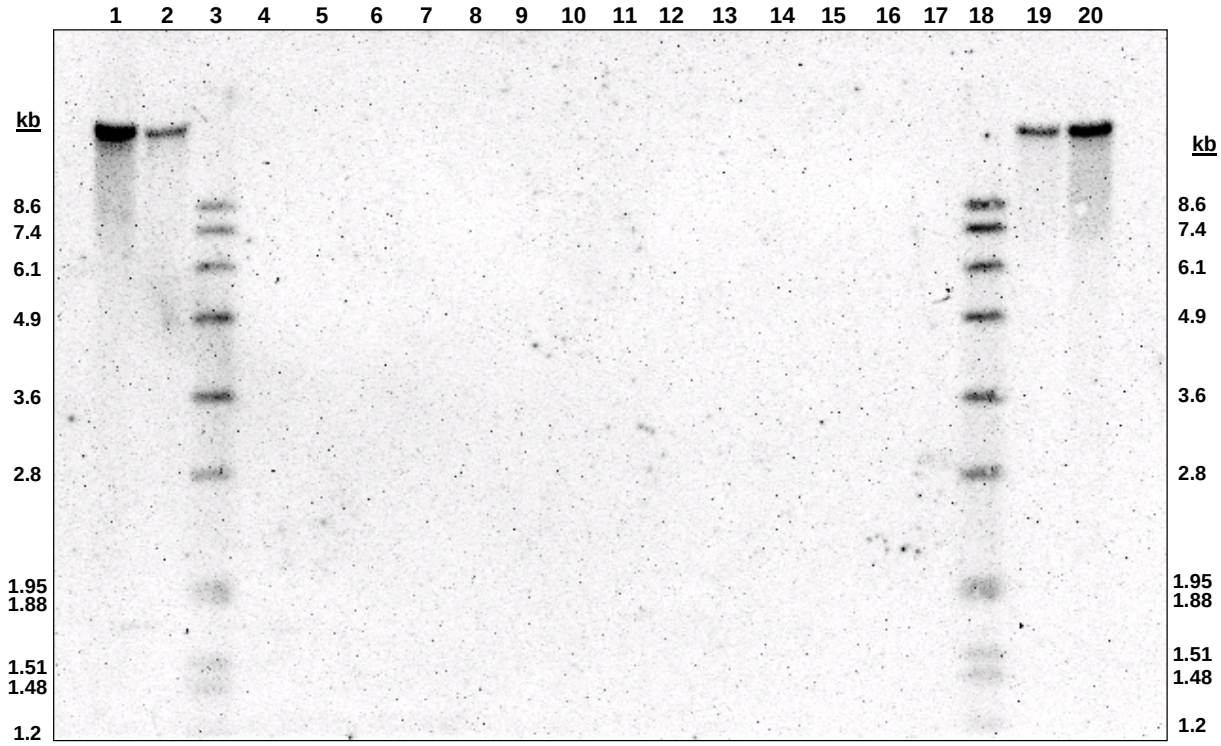


Figure 32. Southern blot analysis of DAS-59122-7; Sac I digest, tet probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the tet gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

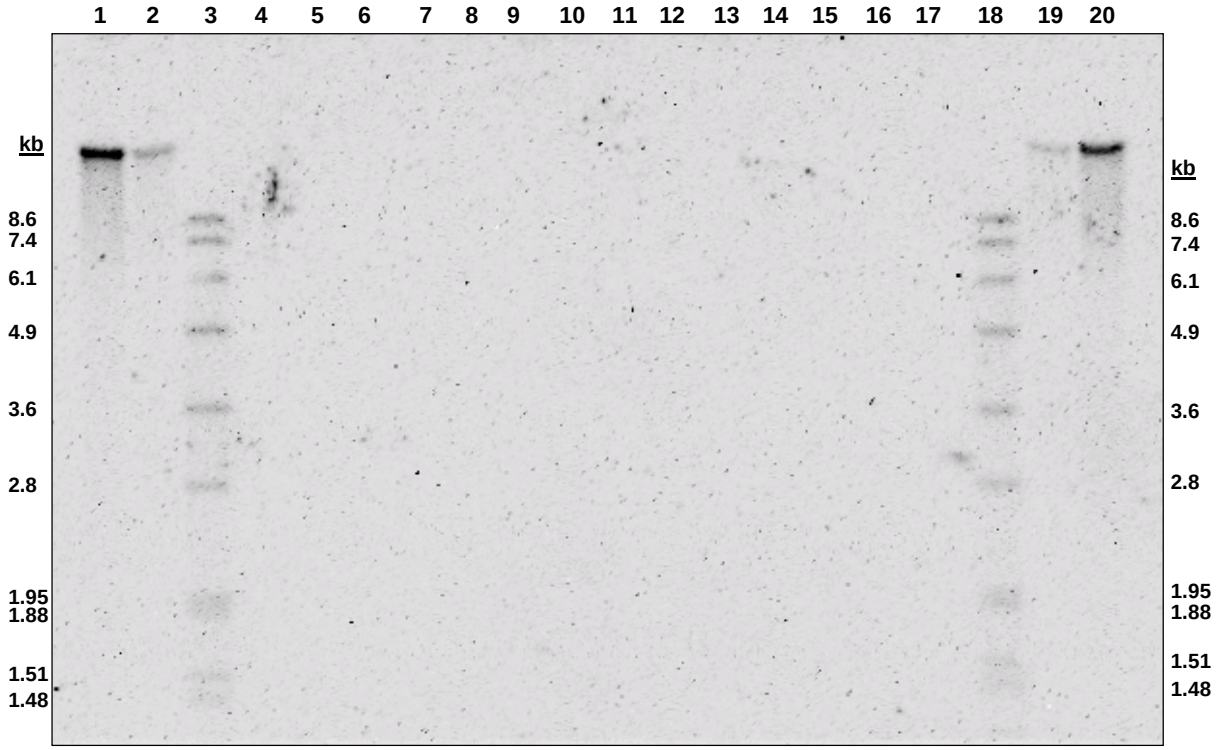


Figure 33. Southern blot analysis of DAS-59122-7; Sac I digest, virG probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the virG gene probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

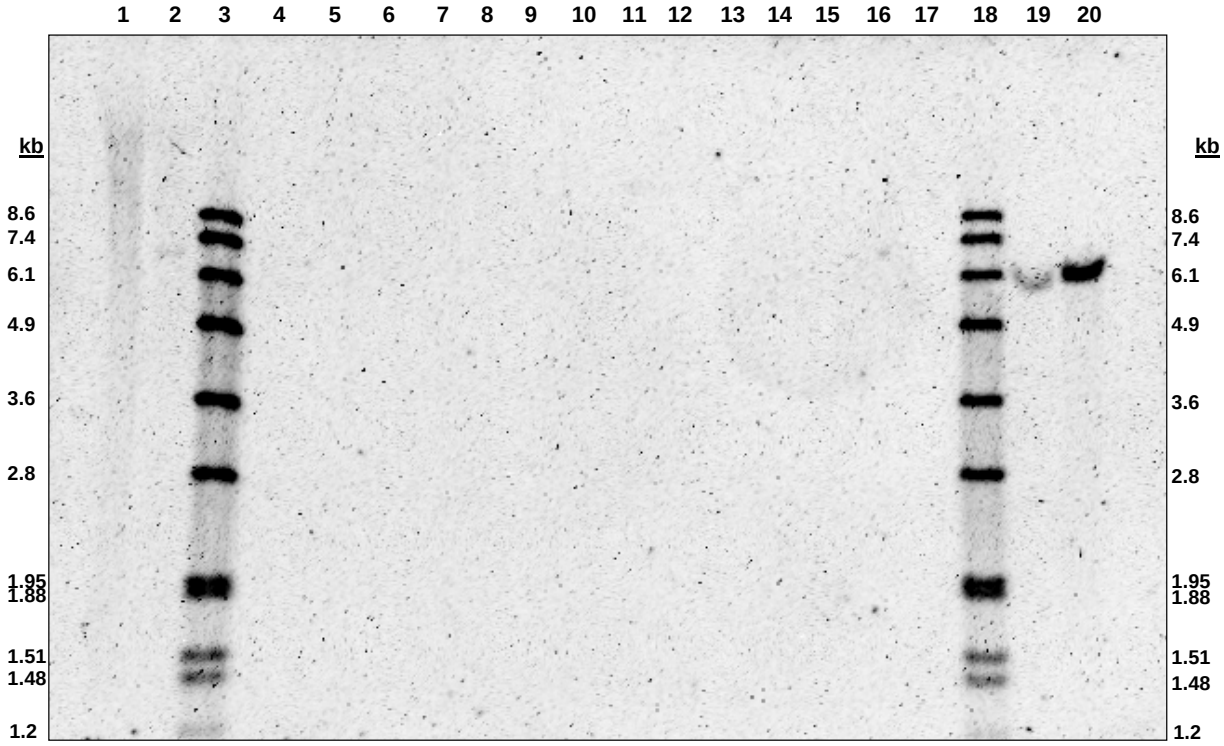


Figure 34. Southern blot analysis of DAS-59122-7; Sac I digest, LB Backbone probe.
DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Sac I and probed with the LB probe. Approximately 7 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 μg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Sac I
2	Hi II + 1 copy PHP17662	Sac I
3	DIG VII + ϕx	
4	05Fx581	Sac I
5	DAS-59122-7-T52 (s) T1S1	Sac I
6	DAS-59122-7-T46 T1S1	Sac I
7	DAS-59122-7-T49 T1S1	Sac I
8	DAS-59122-7-T55 T1S1	Sac I
9	DAS-59122-7-T58 T1S1	Sac I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Sac I
12	DAS-59122-7- T22 BC1	Sac I
13	DAS-59122-7- T23 BC1	Sac I
14	DAS-59122-7- T24 BC1	Sac I
15	DAS-59122-7- T28 BC1	Sac I
16	P38	Sac I
17	PH09B	Sac I
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Sac I
20	P38 + 3 copies PHP17662	Sac I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

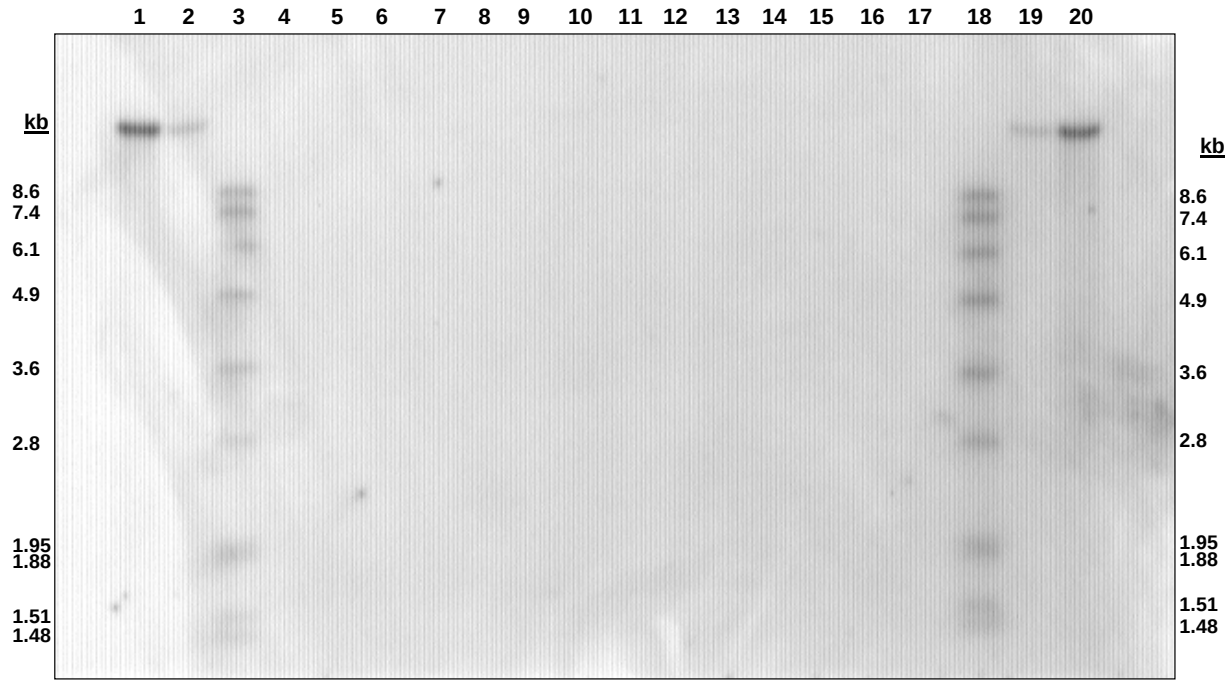


Figure 35. Southern blot analysis of DAS-59122-7; Xho I digest, LB Backbone probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Xho I and probed with the LB probe. Approximately 7 µg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 µg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Xho I
2	Hi II + 1 copy PHP17662	Xho I
3	DIG VII + ϕx	
4	05Fx581	Xho I
5	DAS-59122-7-T52 (s) T1S1	Xho I
6	DAS-59122-7-T58 T1S1	Xho I
7	DAS-59122-7-T49 T1S1	Xho I
8	DAS-59122-7-T55 T1S1	Xho I
9	DAS-59122-7-T59 T1S1	Xho I
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Xho I
12	DAS-59122-7- T22 BC1	Xho I
13	DAS-59122-7- T23 BC1	Xho I
14	DAS-59122-7- T24 BC1	Xho I
15	DAS-59122-7- T28 BC1	Xho I
16	P38	Xho I
17	PH09B	Xho I
18	DIG VII	
19	P38 + 1 copy PHP17662	Xho I
20	P38 + 3 copies PHP17662	Xho I

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

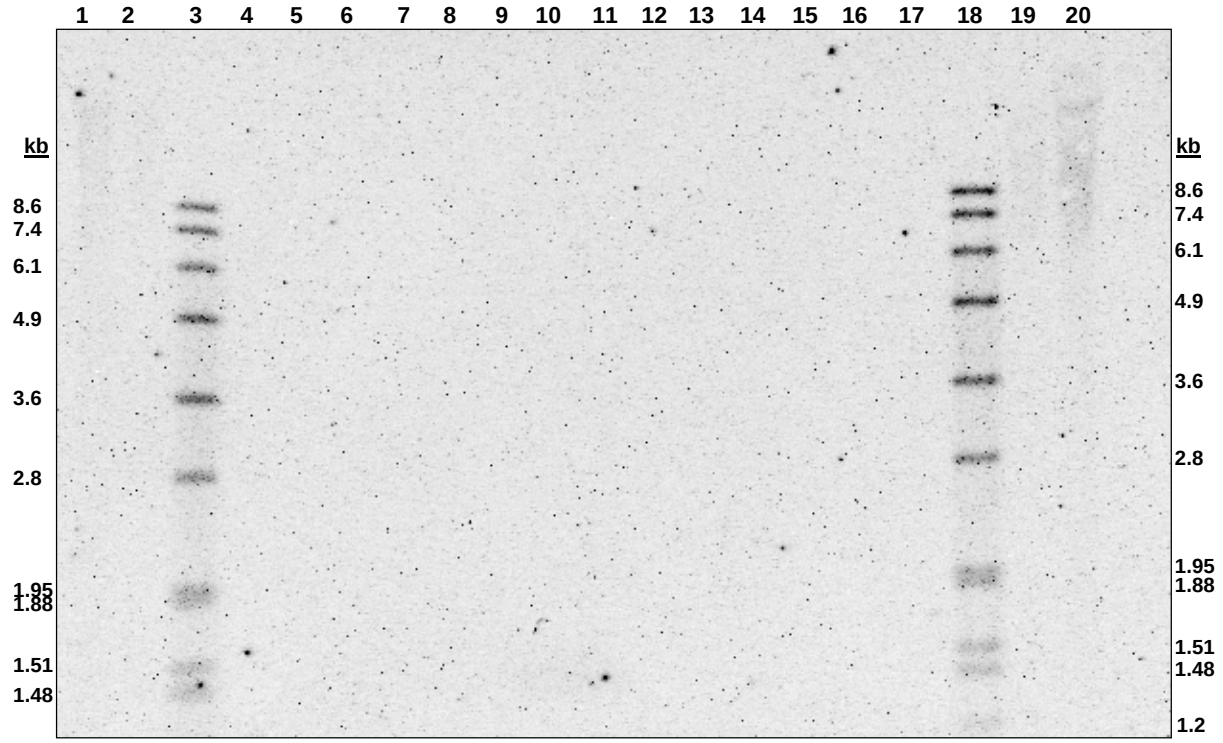
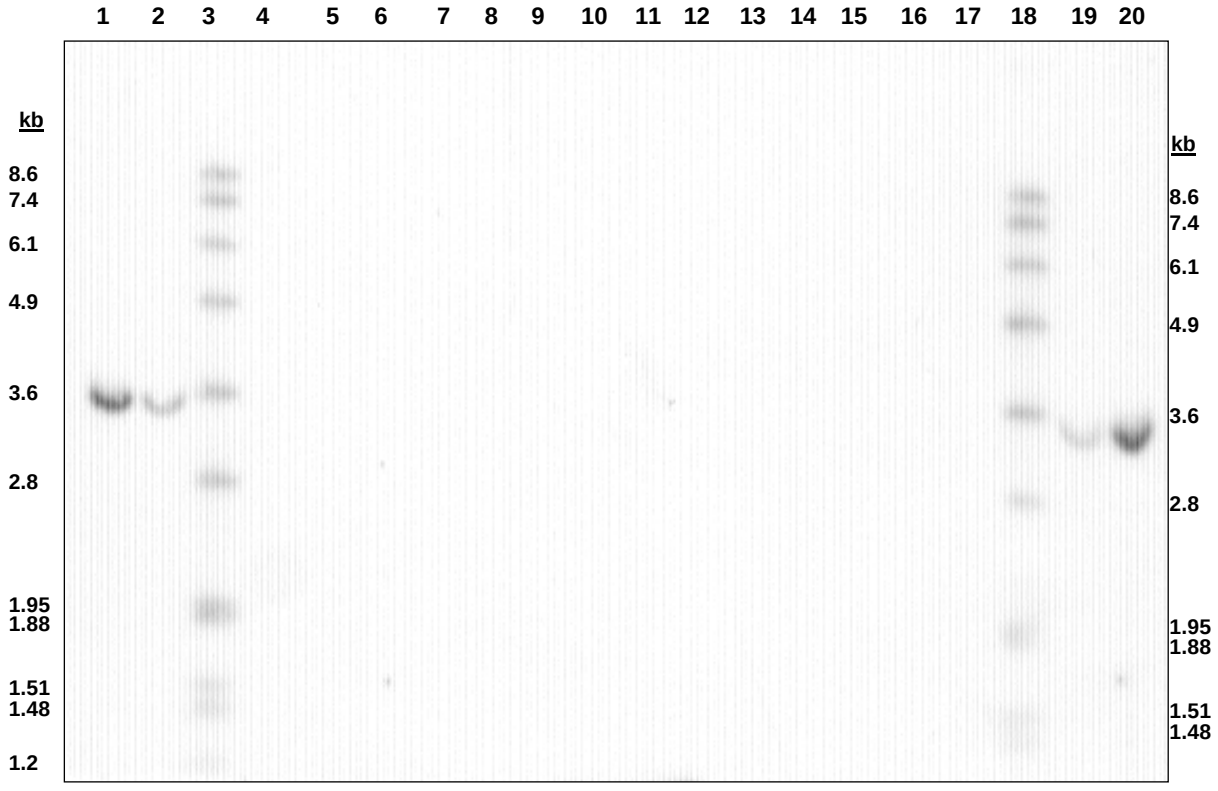


Figure 36. Southern blot analysis of DAS-59122-7; Hind III digest, RB Backbone probe. DNA isolated from transgenic corn event DAS-59122-7 (T1S1 and BC1 generations) and PH09B, P38, Hi-II, and 05Fx581 unmodified corn was digested with Hind III and probed with the RB probe. Approximately 7 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 7 μg of unmodified Hi-II or P38 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane	Sample	Enzyme
1	Hi II + 3 copies PHP17662	Hind III
2	Hi II + 1 copy PHP17662	Hind III
3	DIG VII + ϕx	
4	05Fx581	Hind III
5	DAS-59122-7-T52 (s) T1S1	Hind III
6	DAS-59122-7-T46 T1S1	Hind III
7	DAS-59122-7-T49 T1S1	Hind III
8	DAS-59122-7-T55 T1S1	Hind III
9	DAS-59122-7-T59 T1S1	Hind III
10	Empty	

Lane	Sample	Enzyme
11	DAS-59122-7- T26 (s) BC1	Hind III
12	DAS-59122-7- T22 BC1	Hind III
13	DAS-59122-7- T23 BC1	Hind III
14	DAS-59122-7- T24 BC1	Hind III
15	DAS-59122-7- T28 BC1	Hind III
16	P38	Hind III
17	PH09B	Hind III
18	DIG VII + ϕx	
19	P38 + 1 copy PHP17662	Hind III
20	P38 + 3 copies PHP17662	Hind III

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.



The Southern blot data indicated that the insert in *B.t.* Cry34/35Ab1 event DAS-59122-7 resulted from a single insertion of one intact copy of the T-DNA region from plasmid PHP17662 at one locus in the corn genome. A detailed map of the insertion has been hypothesized and sites for *Sac* I, *Nco* I, *Xho* I, and *Bsa* I were placed relative to the Right and Left Border regions of the T-DNA insertion. In addition, the results did not indicate that rearrangements of the T-DNA had occurred, as all internal restriction enzyme sites for *Hind* III, *Nco* I, and *Sac* I appeared to be intact and produced hybridizing fragments of the expected size. The absence of the tetracycline and spectinomycin resistance genes, the *vir*G gene, and vector backbone DNA regions immediately outside of the Left and Right T-DNA borders was confirmed and suggested that only DNA contained within the T-DNA borders of plasmid PHP17662 was integrated into *B.t.* Cry34/35Ab1 Event DAS-59122-7. Identical fragment sizes were observed in all cases for individual plants from four distinct generations of *B.t.* Cry34/35Ab1 event DAS-59122-7, indicating stability of inheritance across and within generations.

V.B. STABILITY OF THE GENE INSERT

The stability of the inheritance of the gene insert within a generation was investigated via the use of Southern analysis of a single generation of corn plants. The results from this characterization study indicated that the inheritance of the inserted DNA of event DAS-59122-7 is stable within a segregating generation (BC2S1). All 55 individual plants analyzed by Southern blot analysis exhibited a consistent hybridization pattern with each of the gene probes indicating the insertion was equivalent in all individuals within the generation. All null segregants did not show the presence of the T-DNA insertion, as expected in a segregating population.

V.B.1. MOLECULAR CHARACTERIZATION OF INSERT STABILITY

Southern blot analysis was used to determine the genetic equivalence of the inserted DNA within a single generation of transgenic plants (Weber and Igo, 2003). For this analysis, event DAS-59122-7 BC2S1 seed was used. The DAS-59122-7 BC2S1 seed represents transformation into a Hi-II background followed by an outcross for one generation to inbred line PH09B, the resulting F1 was crossed and then backcrossed twice to inbred 581 to make BC2. The final generation represented here is a self-pollination (S1) of the BC2 creating a population that segregates with a ratio of 3 plants containing the introduced genes to 1 plant without the insertion. Seeds were planted and grown in the greenhouse and germinated plants were tested for expression of Cry34Ab1 by lateral flow immunoassay device specific for the Cry34Ab1 protein and by leaf painting with glufosinate-ammonium herbicide to determine the plants expressing PAT protein (resistant to the herbicide) and to also identify the null segregants that do not express either protein. Seeds from three unmodified corn lines; 581, PH09B, and Hi-II were planted and used as negative controls. The unmodified control seeds were representative of the genetic background in event DAS-59122-7 seeds but the control seeds were not transformed and therefore, did not carry any of the genes of interest

(*cry*34Ab1, *cry*35Ab1 or *pat*). Genomic DNA samples used for Southern blot analysis were prepared from leaf tissue harvested from the event DAS-59122-7 plants (BC2S1 generation plants positive for Cry34Ab1 and PAT expression), null segregant plants of the BC2S1 generation, and from unmodified control plants. Plasmid DNA from plasmid PHP17662 (the plasmid used to generate event DAS-59122-7) was used as a positive control to verify probe hybridization. Commercially available DNA molecular weight markers were used as size standards for Southern blot analysis.

Genomic DNA samples prepared from leaf tissue from a total of 55 postive individuals and 23 null segregants from the DAS-59122-7 BC2S1 generation were digested with the *Sac* I restriction enzyme to characterize the event and determine equivalency of the insertion in all individuals within a single generation. Appropriate background controls and positive controls were included on the same Southern blot. The restriction enzyme *Sac* I was selected to provide a comprehensive assessment of unique border junctions and internal fragments for this generation of event DAS-59122-7. Expected results from the *cry*34Ab1 probe would characterize the junction of the Right Border of the T-DNA with the corn genome by hybridizing to a fragment greater than 3217 base pairs (bp) (*Sac* I site at bp position 3217, Figure 2; Figure 37, Table 9). To examine internal *Sac* I sites within the T-DNA, two internal fragments of the expected sizes of 1941 bp (bp position 3217 to 5158, Figure 2; Figure 37, Table 9) and of 1855 bp (bp position 5281 to 7136, Figure 2; Figure 37, Table 9) would be detected with the *cry*35Ab1 probe. The 1855 bp internal fragment would also be detected by the *pat* probe.

Figure 37. Map of T-DNA region from plasmid PHP17662. Map of T-DNA region from plasmid PHP17662 indicating restriction enzyme sites for *Sac* I, and coding regions for the *cry*34Ab1, *cry*35Ab1, and *pat* genes. Probe locations relative to the location of the genes on the T-DNA are indicated as boxes beneath the T-DNA map. All probes were designed to essentially cover the full-length of the target gene sequence.

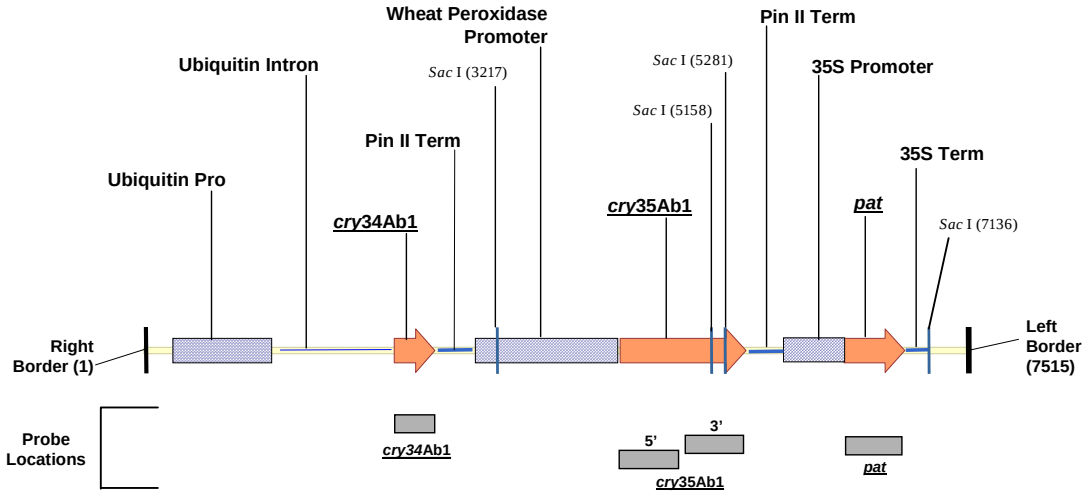


Table 9. Expected and observed hybridization fragments on Southern Blots with gene element probes, event DAS-59122-7

DNA probe	Restriction Enzymes	Expected Fragment Sizes (bp)[1]	Observed Fragment Sizes (bp)[2]	Figure
*cry*34Ab1	*Sac* I	>3217	~3400	38
*cry*34Ab1	*Sac* I	>3217	~3400	39
*cry*34Ab1	*Sac* I	>3217	~3400	40
*cry*34Ab1	*Sac* I	>3217	~3400	41
*cry*35Ab1	*Sac* I	1941 1855 123[3]	1941[4] 1855[4]	42
*cry*35Ab1	*Sac* I	1941 1855 123[3]	1941[4] 1855[4]	43
*cry*35Ab1	*Sac* I	1941 1855 123[3]	1941[4] 1855[4]	44
*cry*35Ab1	*Sac* I	1941 1855 123[3]	1941[4] 1855[4]	45
pat	*Sac* I	1855	1855[4]	46
pat	*Sac* I	1855	1855[4]	47
pat	*Sac* I	1855	1855[4]	48
pat	*Sac* I	1855	1855[4]	49

1. Expected fragment sizes are based on the T-DNA map of PHP17662 as shown in Figure 37.
2. Observed fragments sizes are considered approximate from these analyses and are based on the indicated sizes of the DIG-labeled DNA Molecular Weight Marker VII fragments. Due to incorporation of DIG molecules for visualization, the marker fragments typically run approximately 5–10% larger than their actual indicated molecular weight.
3. The expected 123 bp fragment was not detected, as fragments below approximately 700 bp were run off the gel during electrophoresis and were not transferred to the nylon membrane.
4. Observed size of fragment or fragments is presumed to be the same as expected due to equivalent migration with bands in positive plasmid control PHP17662 lane.

The expected and observed sizes of hybridizing fragments from the Southern blot analysis conducted on this generation are summarized in Table 9. Fifty-five (55) positive plants analyzed from this generation showed the presence of hybridizing bands when probed with *cry*34Ab1, *cry*35Ab1, and *pat* probes (Figures 38 through 49) and, in each case, correlated with the presence of Cry34Ab1 and PAT expression (Tables 10, 11, 12,

and 13). The 23 null segregants analyzed did not hybridize to the three gene probes and this correlated with the absence of Cry34Ab1 and PAT expression (Tables 10, 11, 12, and 13). In addition, each of the probes hybridized to the same respective band(s) in each individual plant analyzed, indicating that within the generation all individual plants contained the same insertion and were equivalent to one another. The *cry*34Ab1 probe hybridized to the expected border fragment of approximately 3400 bp in all 55 individual plants tested and the fragment was absent in all null segregants (Table 9, Figures 38, 39, 40, and 41). The *cry*35Ab1 probe hybridized to the expected internal 1941 bp and 1855 bp fragments in the 55 individual plants and no bands were detected in the null segregants (Table 9, Figures 42, 43, 44, and 45). The *pat* probe hybridized to the expected 1855 bp internal fragment in the 55 individual plants and the band was absent in the null segregants (Table 9, Figures 46, 47, 48, and 49). All results correlated with the previous Southern analyses on different generations of DAS-59122-7 (Locke and Igo, 2003; Locke *et al.*, 2003; Appendix 2, Sections 6 and 7) indicating a single intact T-DNA insertion in the event. A summary map of the T-DNA insertion region with the location of *Sac* I restriction enzyme sites located internally or in the corn genome is presented in Figure 50 and is supported by data from these two previous studies (Locke and Igo, 2003; Locke *et al.*, 2003; Appendix 2, Sections 6 and 7). Taken together, this Southern analysis and those studies referenced previously show the genetic stability of the insertion in event DAS-59122-7 multiple individual plants over several different generations.

Table 10. Summary of Cry34Ab1 and PAT protein expression and Southern hybridization data for Gel 1 — event DAS-59122-7

Lane	Sample	Cry34Ab1 and PAT Expression[1]	Southern Blot Figure 38 *cry*34Ab1 Probe[2]	Southern Blot Figure 42 *cry*35Ab1 Probe[2]	Southern Blot Figure 46 *pat* Probe[2]
1	PH09B + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
2	PH09B + 1 copy PHP17662 (Plasmid Control)	N/A	+	+	+
3	DIG VII	N/A			
4	PH09B (Control)	Negative	-	-	-
5	DAS-59122-7-T1	Positive	+	+	+
6	DAS-59122-7-T3	Positive	+	+	+
7	DAS-59122-7-T4	Positive	+	+	+
8	DAS-59122-7-T6	Positive	+	+	+
9	DAS-59122-7-T8	Positive	+	+	+
10	DAS-59122-7-T9	Positive	+	+	+
11	DAS-59122-7-T10	Positive	+	+	+
12	DAS-59122-7-T12	Positive	+	+	+
13	DAS-59122-7-T13	Positive	+	+	+
14	DAS-59122-7-T14	Positive	+	+	+
15	DAS-59122-7-T15	Positive	+	+	+
16	DAS-59122-7-T16	Positive	+	+	+
17	DAS-59122-7-T17	Positive	+	+	+
18	DAS-59122-7-T18	Positive	+	+	+
19	DAS-59122-7-T19	Positive	+	+	+
20	DAS-59122-7-T20	Positive	+	+	+
21	DAS-59122-7-T21	Positive	+	+	+
22	DAS-59122-7-T25	Positive	+	+	+
23	DAS-59122-7-T26	Positive	+	+	+
24	DAS-59122-7-T2	Negative	-	-	-
25	DAS-59122-7-T5	Negative	-	-	-
26	DAS-59122-7-T7	Negative	-	-	-
27	Blank	N/A			
28	DIG VII	N/A			
29	PH09B + 1 copy PHP17662 (Plasmid Control)	N/A	+	+	+
30	PH09B + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+

[1]Positive Cry34Ab1 expression indicates detection of protein expression as determined by the immunoassay-based lateral flow device specific for Cry34Ab1 protein detection. Negative indicates no detection of the Cry34Ab1 protein. Positive PAT expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.
[2]+ indicates hybridization signal on Southern blot; - indicates no hybridization signal on Southern blot.

Table 11. Summary of Cry34Ab1 and PAT protein expression and Southern hybridization data for Gel 2 — event DAS-59122-7.

Lane	Sample	Cry34Ab1 and PAT Expression[1]	Southern Blot Figure 39 *cry*34Ab1 Probe[2]	Southern Blot Figure 43 *cry*35Ab1 Probe[2]	Southern Blot Figure 47 *pat* Probe[2]
1	Hi-II + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+
2	Hi-II + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
3	DIG VII	N/A			
4	Hi-II (Control)	Negative	-	-	-
5	DAS-59122-7-T27	Positive	+	+	+
6	DAS-59122-7-T30	Positive	+	+	+
7	DAS-59122-7-T31	Positive	+	+	+
8	DAS-59122-7-T32	Positive	+	+	+
9	DAS-59122-7-T34	Positive	+	+	+
10	DAS-59122-7-T35	Positive	+	+	+
11	DAS-59122-7-T36	Positive	+	+	+
12	DAS-59122-7-T37	Positive	+	+	+
13	DAS-59122-7-T38	Positive	+	+	+
14	DAS-59122-7-T39	Positive	+	+	+
15	DAS-59122-7-T42	Positive	+	+	+
16	DAS-59122-7-T43	Positive	+	+	+
17	DAS-59122-7-T45	Positive	+	+	+
18	DAS-59122-7-T47	Positive	+	+	+
19	DAS-59122-7-T49	Positive	+	+	+
20	DAS-59122-7-T50	Positive	+	+	+
21	DAS-59122-7-T51	Positive	+	+	+
22	DAS-59122-7-T52	Positive	+	+	+
23	DAS-59122-7-T11	Negative	-	-	-
24	DAS-59122-7-T22	Negative	-	-	-
25	DAS-59122-7-T23	Negative	-	-	-
26	Blank	N/A			
27	Hi-II (Control)	Negative	-	-	-
28	DIG VII	N/A			
29	Hi-II + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
30	Hi-II + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+

[1]Positive Cry34Ab1 expression indicates detection of protein expression as determined by the immunoassay-based lateral flow device specific for Cry34Ab1 protein detection. Negative indicates no detection of the Cry34Ab1 protein. Positive PAT expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.
[2]+ indicates hybridization signal on Southern blot; - indicates no hybridization signal on Southern blot.

Table 12. Summary of Cry34Ab1 and PAT protein expression and southern hybridization data for Gel 3 — event DAS-59122-7

Lane	Sample	Cry34Ab1 and PAT Expression[1]	Southern Blot Figure 40 *cry*34Ab1 Probe[2]	Southern Blot Figure 44 *cry*35Ab1 Probe[2]	Southern Blot Figure 48 *pat* Probe[2]
1	581 + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+
2	581 + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
3	DIG VII	N/A			
4	581 (Control)	Negative	-	-	-
5	Blank	N/A			
6	DAS-59122-7-T54	Positive	+	+	+
7	DAS-59122-7-T55	Positive	+	+	+
8	DAS-59122-7-T56	Positive	+	+	+
9	DAS-59122-7-T58	Positive	+	+	+
10	DAS-59122-7-T59	Positive	+	+	+
11	DAS-59122-7-T60	Positive	+	+	+
12	DAS-59122-7-T61	Positive	+	+	+
13	DAS-59122-7-T62	Positive	+	+	+
14	DAS-59122-7-T63	Positive	+	+	+
15	DAS-59122-7-T68	Positive	+	+	+
16	DAS-59122-7-T69	Positive	+	+	+
17	DAS-59122-7-T72	Positive	+	+	+
18	DAS-59122-7-T73	Positive	+	+	+
19	DAS-59122-7-T76	Positive	+	+	+
20	DAS-59122-7-T77	Positive	+	+	+
21	DAS-59122-7-T78	Positive	+	+	+
22	DAS-59122-7-T79	Positive	+	+	+
23	DAS-59122-7-T80	Positive	+	+	+
24	DAS-59122-7-T28	Negative	-	-	-
25	DAS-59122-7-T33	Negative	-	-	-
26	DAS-59122-7-T40	Negative	-	-	-
27	Blank	N/A			
28	DIG VII	N/A			
29	581 + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
30	581 + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+

[1]Positive Cry34Ab1 expression indicates detection of protein expression as determined by the immunoassay-based lateral flow device specific for Cry34Ab1 protein detection. Negative indicates no detection of the Cry34Ab1 protein. Positive PAT expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.

[2]+ indicates hybridization signal on Southern blot; - indicates no hybridization signal on Southern blot.

Table 13. Summary of Cry34Ab1 and PAT protein expression and southern hybridization data for Gel 4 — event DAS-59122-7

Lane	Sample	Cry34Ab1 and PAT Expression[1]	Southern Blot Figure 41 *cry*34Ab1 Probe[2]	Southern Blot Figure 45 *cry*35Ab1 Probe[2]	Southern Blot Figure 49 *pat* Probe[2]
1	Hi-II + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+
2	Hi-II + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
3	DIG VII	N/A			
4	Hi-II (Control)	Negative	-	-	-
5	Blank	N/A			
6	DAS-59122-7–T56	Positive	+	+	+
7	Blank	N/A			
8	DAS-59122-7–T29	Negative	-	-	-
9	DAS-59122-7–T41	Negative	-	-	-
10	DAS-59122-7–T44	Negative	-	-	-
11	DAS-59122-7–T46	Negative	-	-	-
12	DAS-59122-7–T53	Negative	-	-	-
13	DAS-59122-7–T57	Negative	-	-	-
14	DAS-59122-7–T64	Negative	-	-	-
15	DAS-59122-7–T65	Negative	-	-	-
16	DAS-59122-7–T66	Negative	-	-	-
17	DAS-59122-7–T67	Negative	-	-	-
18	DAS-59122-7–T70	Negative	-	-	-
19	DAS-59122-7–T71	Negative	-	-	-
20	DAS-59122-7–T74	Negative	-	-	-
21	DAS-59122-7–T75	Negative	-	-	-
22	DAS-59122-7–T11	Negative	-	-	-
23	DAS-59122-7–T22	Negative	-	-	-
24	Blank	N/A			
25	DAS-59122-7–T78	Positive	+	+	+
26	Blank	N/A			
27	DIG VII	N/A			
28	581 + 3 copies PHP17662 (Plasmid Control)	N/A	+	+	+
29	581 + 7 copies PHP17662 (Plasmid Control)	N/A	+	+	+
30	Blank	N/A			

[1]Positive Cry34Ab1 expression indicates detection of protein expression as determined by the immunoassay-based lateral flow device specific for Cry34Ab1 protein detection. Negative indicates no detection of the Cry34Ab1 protein. Positive PAT expression indicates plants that exhibited resistance to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.
[2]+ indicates hybridization signal on Southern blot; - indicates no hybridization signal on Southern blot.

Figure 38. Southern blot analysis of Gel 1; *cry*34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B unmodified corn was digested with *Sac* I and probed with the *cry*34Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified PH09B DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	PH09B + 3 copies PHP17662
2	PH09B + 1 copy PHP17662
3	DIG VII
4	PH09B
5	DAS-59122-7–T1
6	DAS-59122-7–T3
7	DAS-59122-7–T4
8	DAS-59122-7–T6
9	DAS-59122-7–T8
10	DAS-59122-7–T9

Lane	Sample
11	DAS-59122-7–T10
12	DAS-59122-7–T12
13	DAS-59122-7–T13
14	DAS-59122-7–T14
15	DAS-59122-7–T15
16	DAS-59122-7–T16
17	DAS-59122-7–T17
18	DAS-59122-7–T18
19	DAS-59122-7–T19
20	DAS-59122-7–T20

Lane	Sample
21	DAS-59122-7–T21
22	DAS-59122-7–T25
23	DAS-59122-7–T26
24	DAS-59122-7–T2 (s)
25	DAS-59122-7–T5 (s)
26	DAS-59122-7–T7 (s)
27	Blank
28	DIG VII
29	PH09B + 1 copy PHP17662
30	PH09B + 3 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

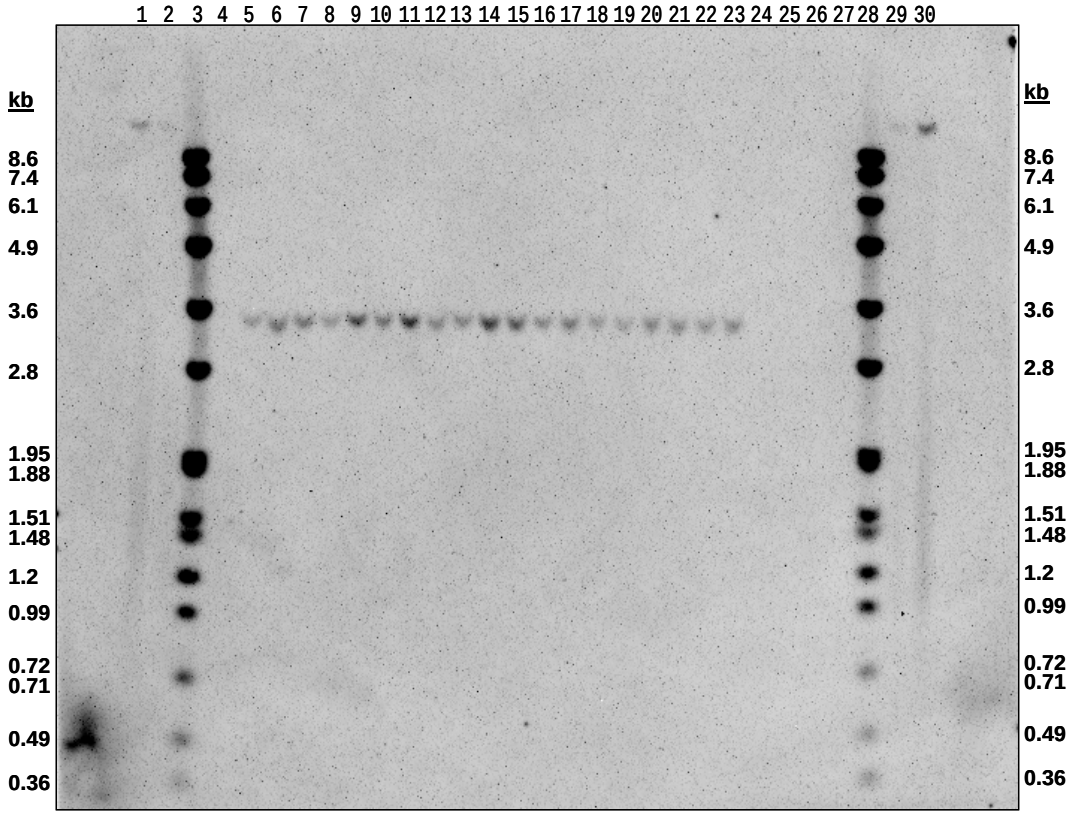


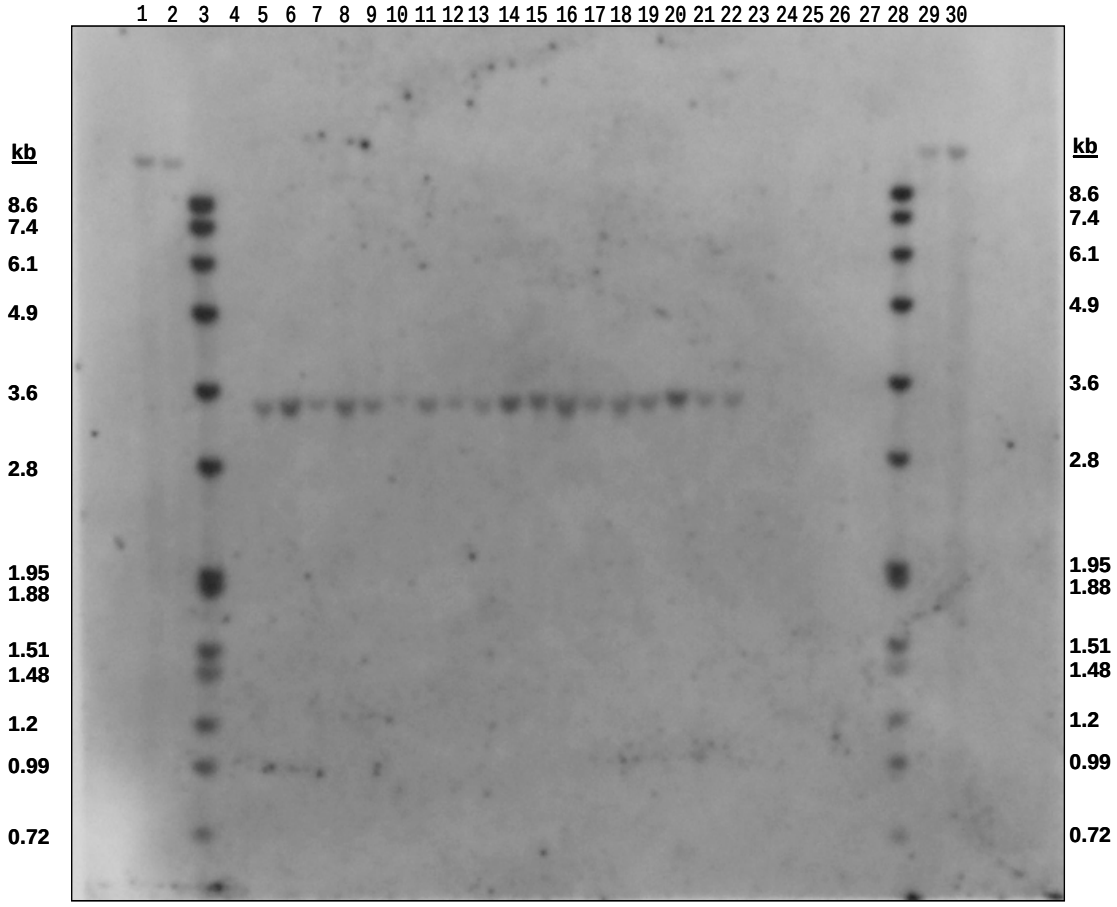
Figure 39. Southern blot analysis of Gel 2; *cry*34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *cry*34Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	Hi-II + 7 copies PHP17662
2	Hi-II + 3 copies PHP17662
3	DIG VII
4	Hi-II
5	DAS-59122-7–T27
6	DAS-59122-7–T30
7	DAS-59122-7–T31
8	DAS-59122-7–T32
9	DAS-59122-7–T34
10	DAS-59122-7–T35

Lane	Sample
11	DAS-59122-7–T36
12	DAS-59122-7–T37
13	DAS-59122-7–T38
14	DAS-59122-7–T39
15	DAS-59122-7–T42
16	DAS-59122-7–T43
17	DAS-59122-7–T45
18	DAS-59122-7–T47
19	DAS-59122-7–T49
20	DAS-59122-7–T50

Lane	Sample
21	DAS-59122-7–T51
22	DAS-59122-7–T52
23	DAS-59122-7–T11 (s)
24	DAS-59122-7–T22 (s)
25	DAS-59122-7–T23 (s)
26	Blank
27	Hi-II
28	DIG VII
29	Hi-II + 3 copies PHP17662
30	Hi-II + 7 copies PHP17662



(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

Figure 40. Southern blot analysis of Gel 3; *cry*34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and 581 unmodified corn was digested with *Sac* I and probed with the *cry*34Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	581 + 7 copies PHP17662
2	581 + 3 copies PHP17662
3	DIG VII
4	581
5	Blank
6	DAS-59122-7–T54
7	DAS-59122-7–T55
8	DAS-59122-7–T56
9	DAS-59122-7–T58
10	DAS-59122-7–T59

Lane	Sample
11	DAS-59122-7–T60
12	DAS-59122-7–T61
13	DAS-59122-7–T62
14	DAS-59122-7–T63
15	DAS-59122-7–T68
16	DAS-59122-7–T69
17	DAS-59122-7–T72
18	DAS-59122-7–T73
19	DAS-59122-7–T76
20	DAS-59122-7–T77

Lane	Sample
21	DAS-59122-7–T78
22	DAS-59122-7–T79
23	DAS-59122-7–T80
24	DAS-59122-7–T28 (s)
25	DAS-59122-7–T33 (s)
26	DAS-59122-7–T40 (s)
27	Blank
28	DIG VII
29	581 + 3 copies PHP17662
30	581 + 7 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

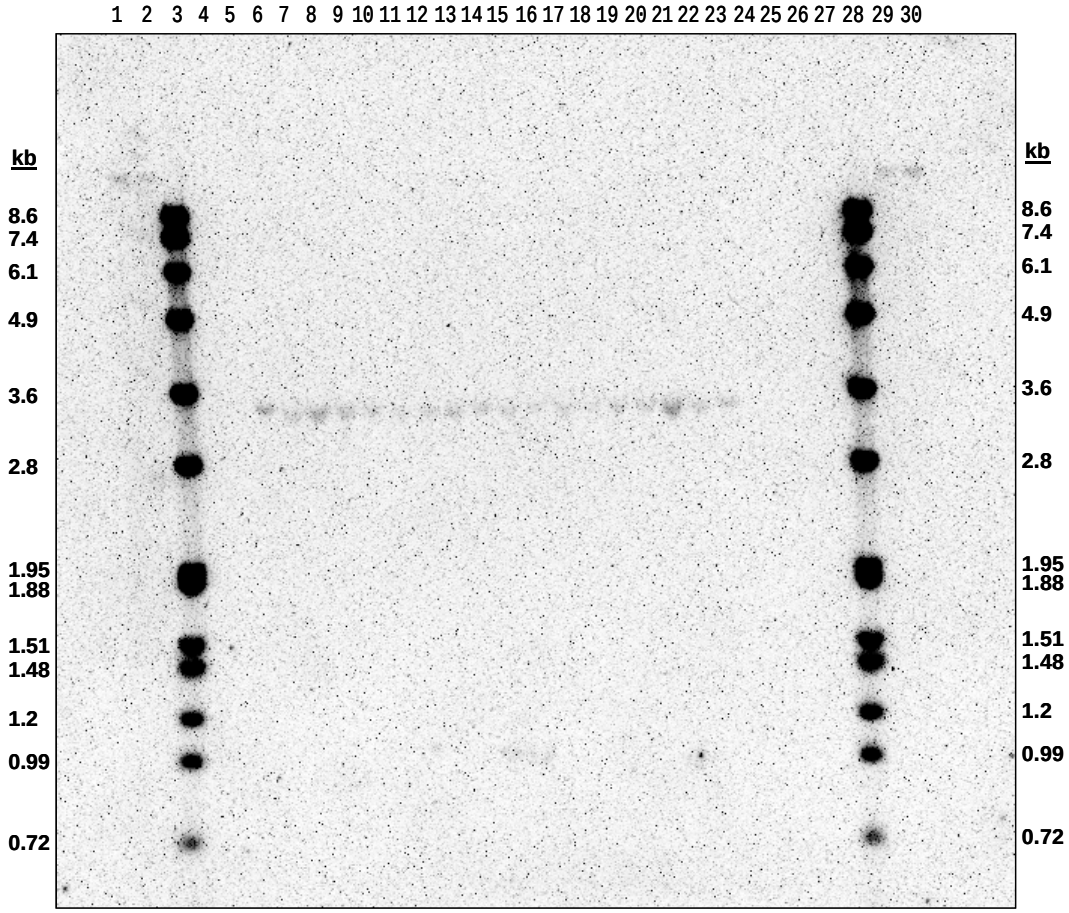


Figure 41. Southern blot analysis of Gel 4; *cry*34Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *cry*34Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of either unmodified Hi-II or 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Approximately 0.5 μg of DAS-59122-7 T66 DNA was loaded in lane 16.

Lane Assignments:

Lane	Sample
1	Hi-II + 7 copies PHP17662
2	Hi-II + 3 copies PHP17662
3	DIG VII
4	Hi-II
5	Blank
6	DAS-59122-7–T56
7	Blank
8	DAS-59122-7–T29 (s)
9	DAS-59122-7–T41 (s)
10	DAS-59122-7–T44 (s)

Lane	Sample
11	DAS-59122-7–T46 (s)
12	DAS-59122-7–T53 (s)
13	DAS-59122-7–T57 (s)
14	DAS-59122-7–T64 (s)
15	DAS-59122-7–T65 (s)
16	DAS-59122-7–T66 (s)
17	DAS-59122-7–T67 (s)
18	DAS-59122-7–T70 (s)
19	DAS-59122-7–T71 (s)
20	DAS-59122-7–T74 (s)

Lane	Sample
21	DAS-59122-7–T75 (s)
22	DAS-59122-7–T11 (s)
23	DAS-59122-7–T22 (s)
24	Blank
25	DAS-59122-7–T78
26	Blank
27	DIG VII
28	581 + 3 copies PHP17662
29	581 + 7 copies PHP17662
30	Blank

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

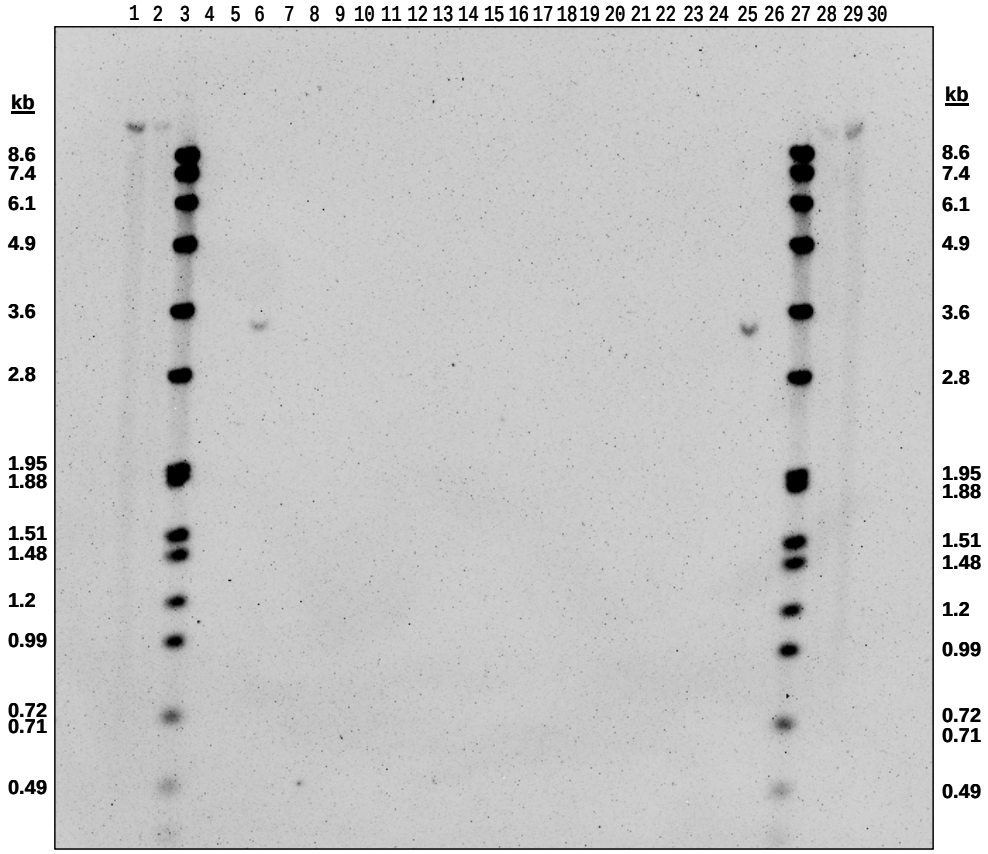


Figure 42. Southern blot analysis of Gel 1; *cry*35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B unmodified corn was digested with *Sac* I and probed with the *cry*35Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified PH09B DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	PH09B + 3 copies PHP17662
2	PH09B + 1 copy PHP17662
3	DIG VII
4	PH09B
5	DAS-59122-7–T1
6	DAS-59122-7–T3
7	DAS-59122-7–T4
8	DAS-59122-7–T6
9	DAS-59122-7–T8
10	DAS-59122-7–T9

Lane	Sample
11	DAS-59122-7–T10
12	DAS-59122-7–T12
13	DAS-59122-7–T13
14	DAS-59122-7–T14
15	DAS-59122-7–T15
16	DAS-59122-7–T16
17	DAS-59122-7–T17
18	DAS-59122-7–T18
19	DAS-59122-7–T19
20	DAS-59122-7–T20

Lane	Sample
21	DAS-59122-7–T21
22	DAS-59122-7–T25
23	DAS-59122-7–T26
24	DAS-59122-7–T2 (s)
25	DAS-59122-7–T5 (s)
26	DAS-59122-7–T7 (s)
27	Blank
28	DIG VII
29	PH09B + 1 copy PHP17662
30	PH09B + 3 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

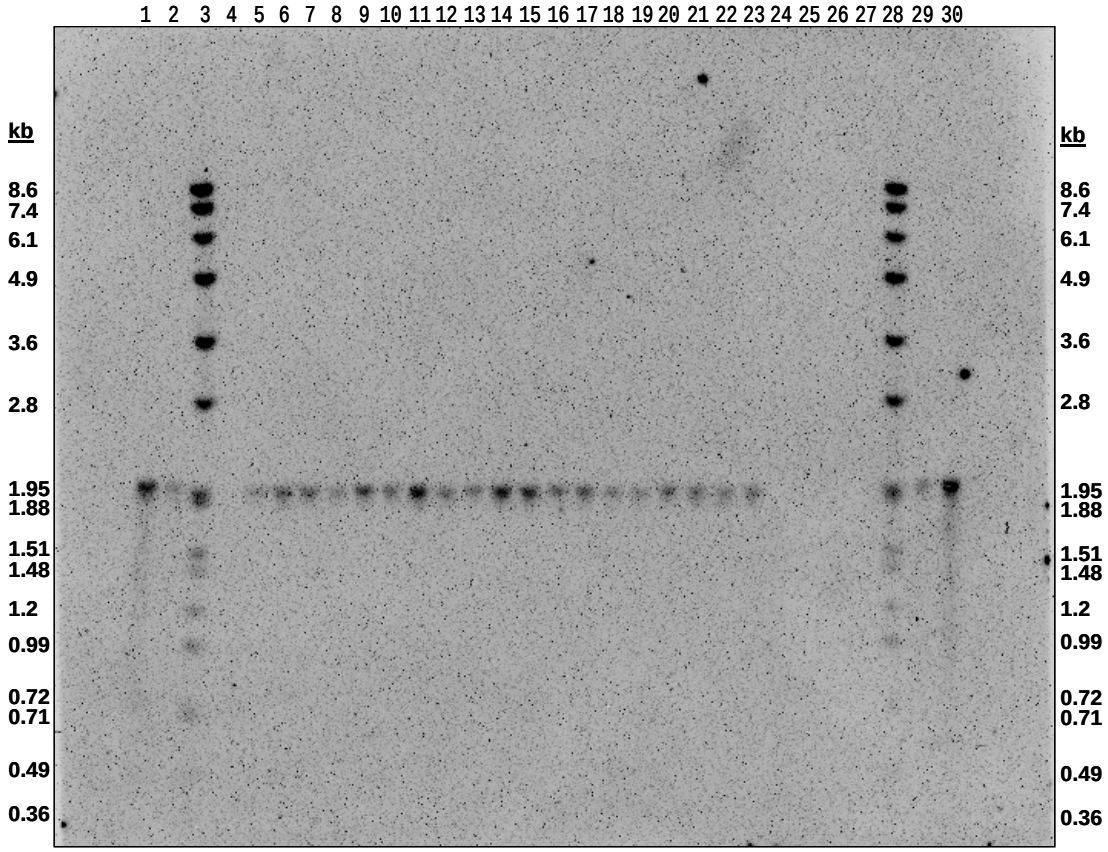


Figure 43. Southern blot analysis of Gel 2; *cry*35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *cry*35Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	Hi-II + 7 copies PHP17662
2	Hi-II + 3 copies PHP17662
3	DIG VII
4	Hi-II
5	DAS-59122-7–T27
6	DAS-59122-7–T30
7	DAS-59122-7–T31
8	DAS-59122-7–T32
9	DAS-59122-7–T34
10	DAS-59122-7–T35

Lane	Sample
11	DAS-59122-7–T36
12	DAS-59122-7–T37
13	DAS-59122-7–T38
14	DAS-59122-7–T39
15	DAS-59122-7–T42
16	DAS-59122-7–T43
17	DAS-59122-7–T45
18	DAS-59122-7–T47
19	DAS-59122-7–T49
20	DAS-59122-7–T50

Lane	Sample
21	DAS-59122-7–T51
22	DAS-59122-7–T52
23	DAS-59122-7–T11 (s)
24	DAS-59122-7–T22 (s)
25	DAS-59122-7–T23 (s)
26	Blank
27	Hi-II
28	DIG VII
29	Hi-II + 3 copies PHP17662
30	Hi-II + 7 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

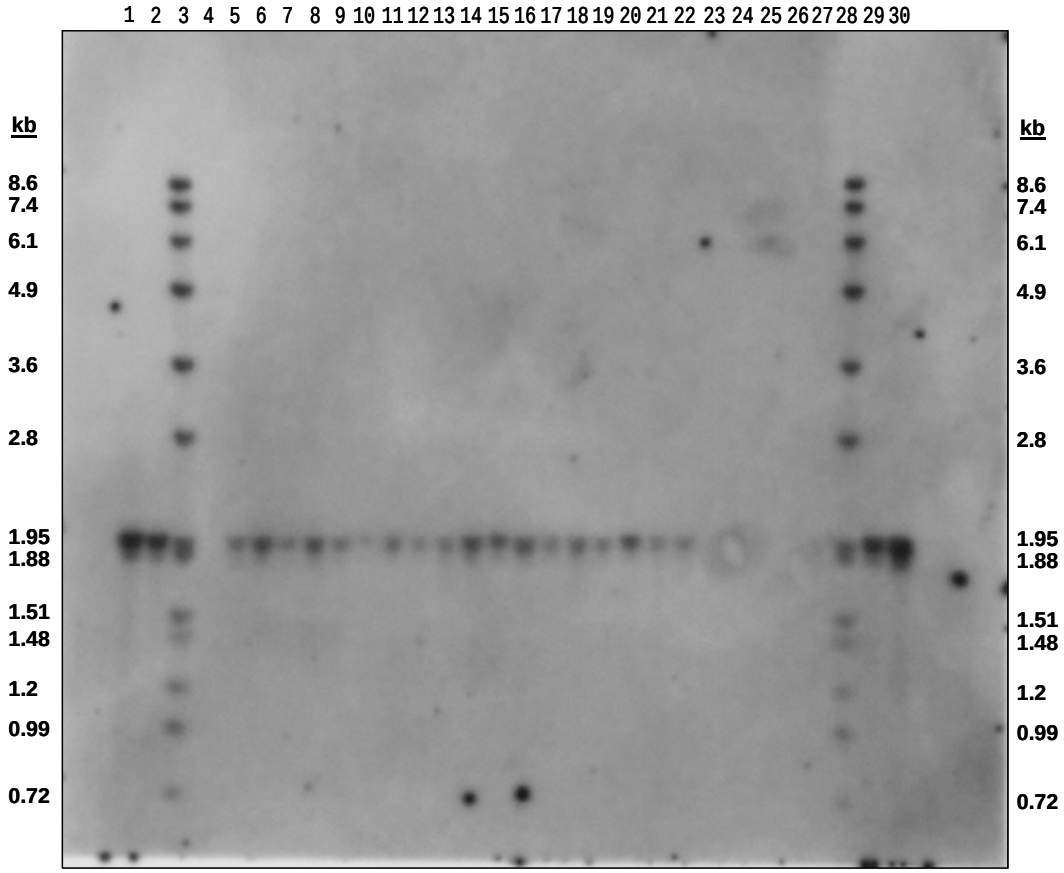


Figure 44. Southern blot analysis of Gel 3; *cry*35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and 581 unmodified corn was digested with *Sac* I and probed with the *cry*35Ab1 gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	581 + 7 copies PHP17662
2	581 + 3 copies PHP17662
3	DIG VII
4	581
5	Blank
6	DAS-59122-7–T54
7	DAS-59122-7–T55
8	DAS-59122-7–T56
9	DAS-59122-7–T58
10	DAS-59122-7–T59

Lane	Sample
11	DAS-59122-7–T60
12	DAS-59122-7–T61
13	DAS-59122-7–T62
14	DAS-59122-7–T63
15	DAS-59122-7–T68
16	DAS-59122-7–T69
17	DAS-59122-7–T72
18	DAS-59122-7–T73
19	DAS-59122-7–T76
20	DAS-59122-7–T77

Lane	Sample
21	DAS-59122-7–T78
22	DAS-59122-7–T79
23	DAS-59122-7–T80
24	DAS-59122-7–T28 (s)
25	DAS-59122-7–T33 (s)
26	DAS-59122-7–T40 (s)
27	Blank
28	DIG VII
29	581 + 3 copies PHP17662
30	581 + 7 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

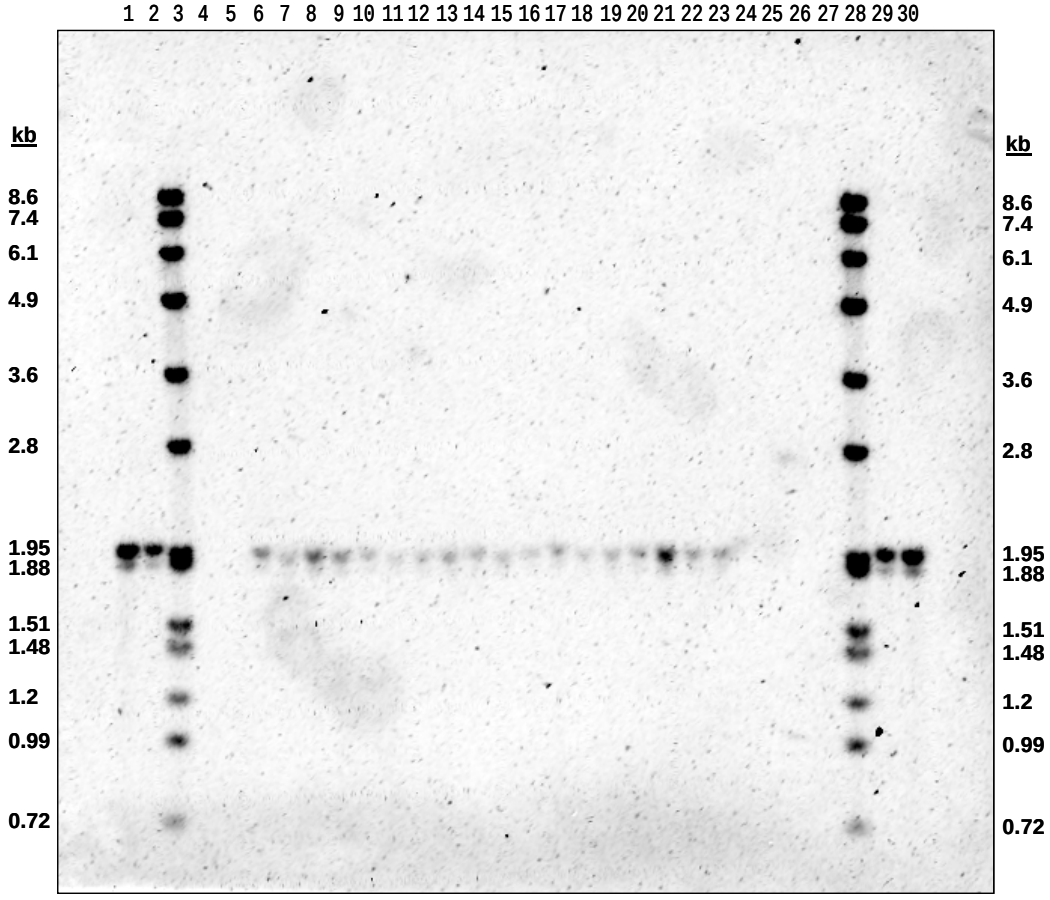


Figure 45. Southern blot analysis of Gel 4; *cry*35Ab1 probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *cry*35Ab1 gene probe. Approximately 3 g of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 g of either unmodified Hi-II or 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Approximately 0.5 g of DAS-59122-7 T66 DNA was loaded in lane 16.

Lane Assignments:

Lane	Sample	Lane	Sample	Lane	Sample
1	Hi-II + 7 copies PHP17662	11	DAS-59122-7–T46 (s)	21	DAS-59122-7–T75 (s)
2	Hi-II + 3 copies PHP17662	12	DAS-59122-7–T53 (s)	22	DAS-59122-7–T11 (s)
3	DIG VII	13	DAS-59122-7–T57 (s)	23	DAS-59122-7–T22 (s)
4	Hi-II	14	DAS-59122-7–T64 (s)	24	Blank
5	Blank	15	DAS-59122-7–T65 (s)	25	DAS-59122-7–T78
6	DAS-59122-7–T56	16	DAS-59122-7–T66 (s)	26	Blank
7	Blank	17	DAS-59122-7–T67 (s)	27	DIG VII
8	DAS-59122-7–T29 (s)	18	DAS-59122-7–T70 (s)	28	581 + 3 copies PHP17662
9	DAS-59122-7–T41 (s)	19	DAS-59122-7–T71 (s)	29	581 + 7 copies PHP17662
10	DAS-59122-7–T44 (s)	20	DAS-59122-7–T74 (s)	30	Blank

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

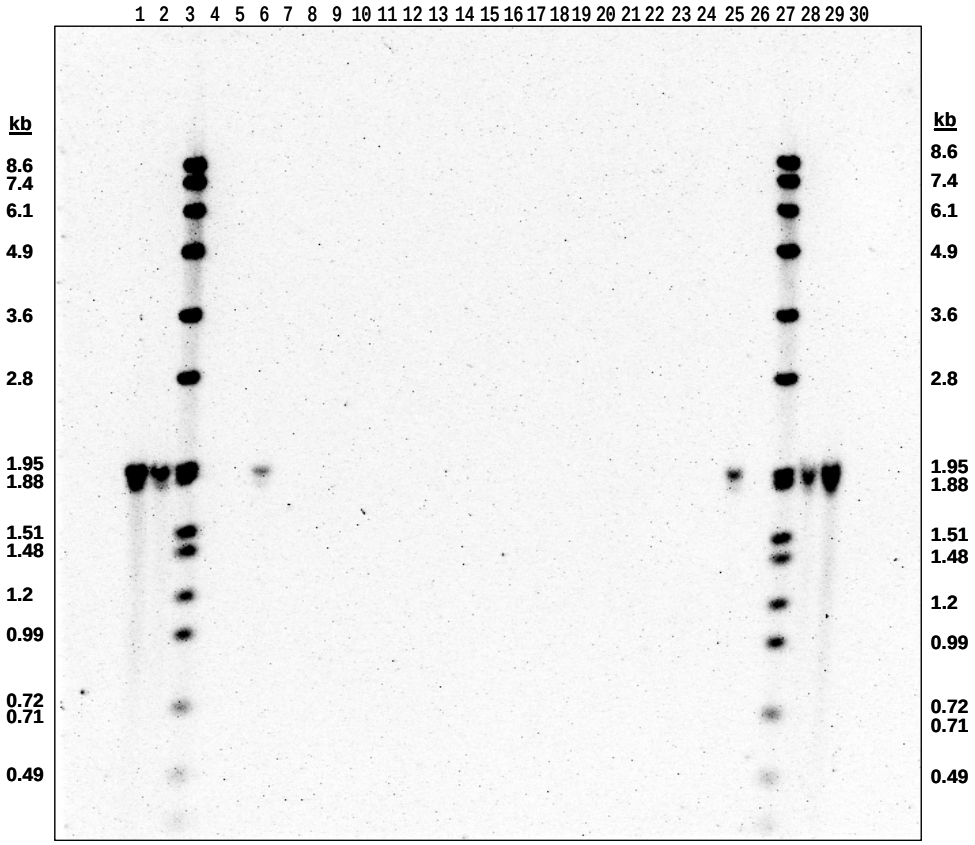


Figure 46. Southern blot analysis of Gel 1; *pat* probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and PH09B unmodified corn was digested with *Sac* I and probed with the *pat* gene probe. Approximately 3 g of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 g of unmodified PH09B DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	PH09B + 3 copies PHP17662
2	PH09B + 1 copy PHP17662
3	DIG VII
4	PH09B
5	DAS-59122-7–T1
6	DAS-59122-7–T3
7	DAS-59122-7–T4
8	DAS-59122-7–T6
9	DAS-59122-7–T8
10	DAS-59122-7–T9

Lane	Sample
11	DAS-59122-7–T10
12	DAS-59122-7–T12
13	DAS-59122-7–T13
14	DAS-59122-7–T14
15	DAS-59122-7–T15
16	DAS-59122-7–T16
17	DAS-59122-7–T17
18	DAS-59122-7–T18
19	DAS-59122-7–T19
20	DAS-59122-7–T20

Lane	Sample
21	DAS-59122-7–T21
22	DAS-59122-7–T25
23	DAS-59122-7–T26
24	DAS-59122-7–T2 (s)
25	DAS-59122-7–T5 (s)
26	DAS-59122-7–T7 (s)
27	Blank
28	DIG VII
29	PH09B + 1 copy PHP17662
30	PH09B + 3 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

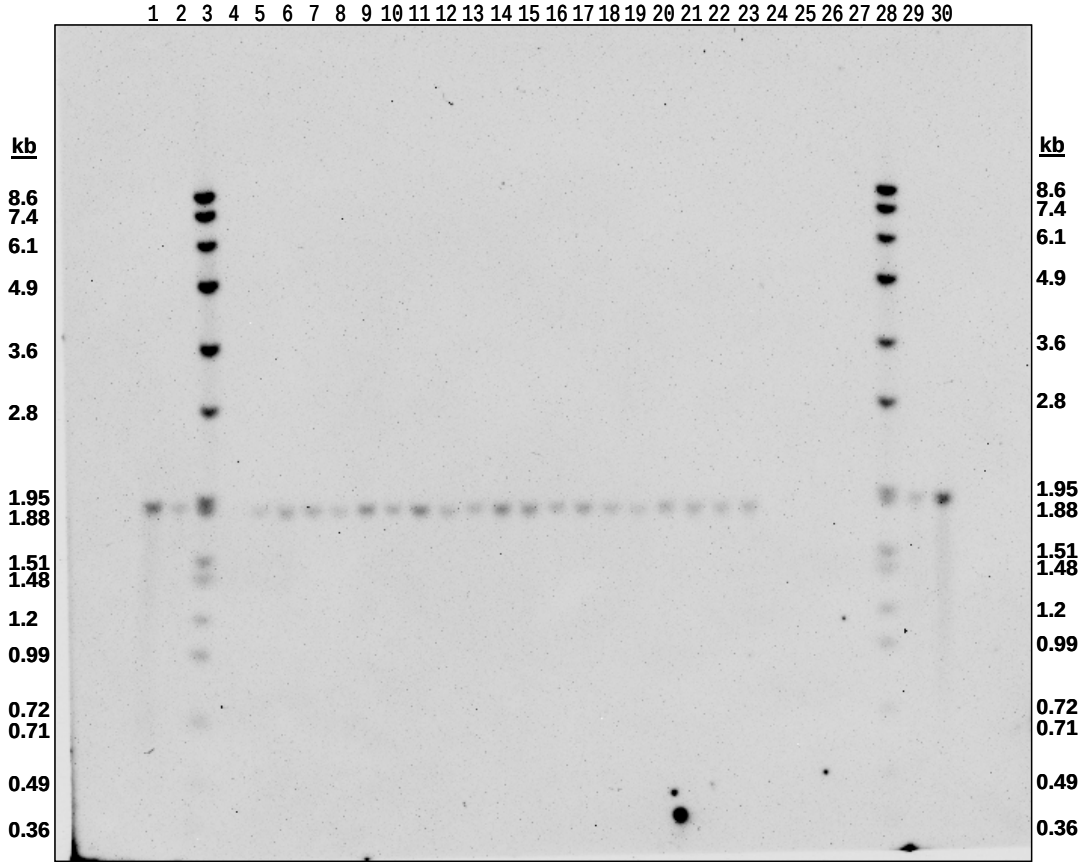


Figure 47. Southern blot analysis of Gel 2; *pat* probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *pat* gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified Hi-II DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	Hi-II + 7 copies PHP17662
2	Hi-II + 3 copies PHP17662
3	DIG VII
4	Hi-II
5	DAS-59122-7–T27
6	DAS-59122-7–T30
7	DAS-59122-7–T31
8	DAS-59122-7–T32
9	DAS-59122-7–T34
10	DAS-59122-7–T35

Lane	Sample
11	DAS-59122-7–T36
12	DAS-59122-7–T37
13	DAS-59122-7–T38
14	DAS-59122-7–T39
15	DAS-59122-7–T42
16	DAS-59122-7–T43
17	DAS-59122-7–T45
18	DAS-59122-7–T47
19	DAS-59122-7–T49
20	DAS-59122-7–T50

Lane	Sample
21	DAS-59122-7–T51
22	DAS-59122-7–T52
23	DAS-59122-7–T11 (s)
24	DAS-59122-7–T22 (s)
25	DAS-59122-7–T23 (s)
26	Blank
27	Hi-II
28	DIG VII
29	Hi-II + 3 copies PHP17662
30	Hi-II + 7 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

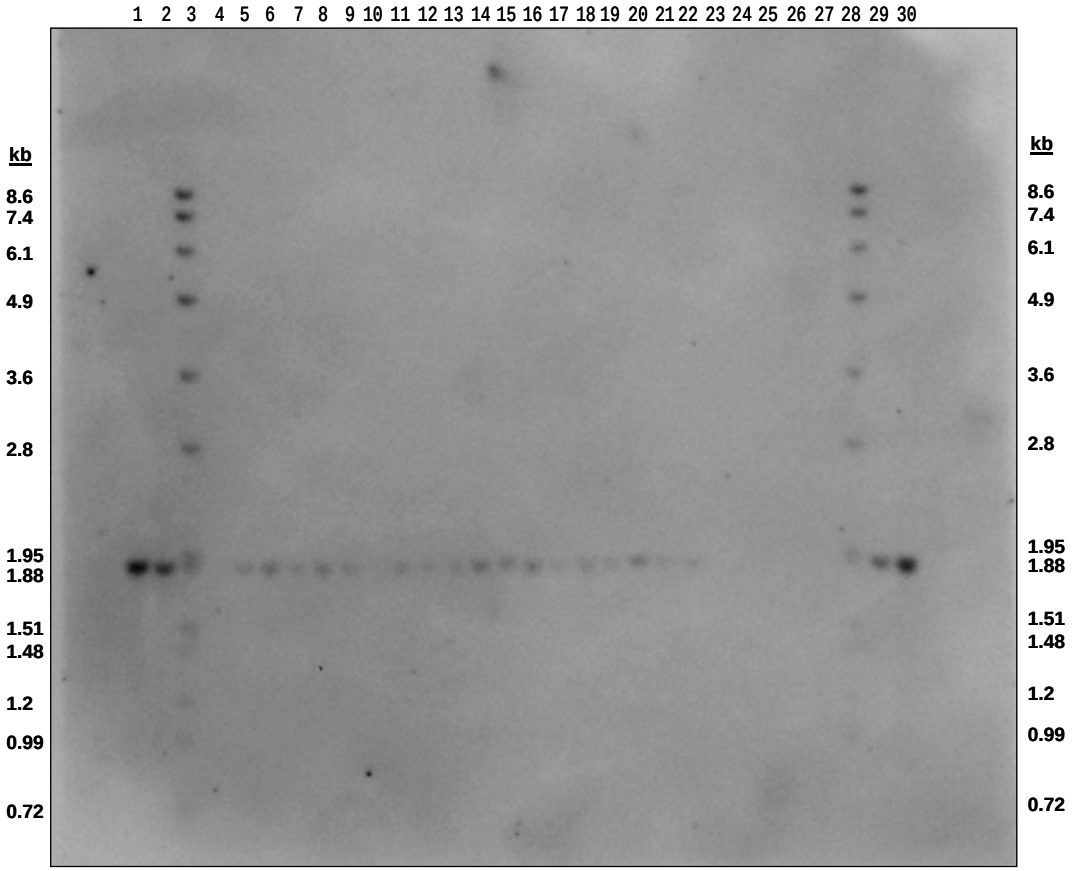


Figure 48. Southern blot analysis of Gel 3; *pat* probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and 581 unmodified corn was digested with *Sac* I and probed with the *pat* gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of unmodified 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image.

Lane Assignments:

Lane	Sample
1	581 + 7 copies PHP17662
2	581 + 3 copies PHP17662
3	DIG VII
4	581
5	Blank
6	DAS-59122-7–T54
7	DAS-59122-7–T55
8	DAS-59122-7–T56
9	DAS-59122-7–T58
10	DAS-59122-7–T59

Lane	Sample
11	DAS-59122-7–T60
12	DAS-59122-7–T61
13	DAS-59122-7–T62
14	DAS-59122-7–T63
15	DAS-59122-7–T68
16	DAS-59122-7–T69
17	DAS-59122-7–T72
18	DAS-59122-7–T73
19	DAS-59122-7–T76
20	DAS-59122-7–T77

Lane	Sample
21	DAS-59122-7–T78
22	DAS-59122-7–T79
23	DAS-59122-7–T80
24	DAS-59122-7–T28 (s)
25	DAS-59122-7–T33 (s)
26	DAS-59122-7–T40 (s)
27	Blank
28	DIG VII
29	581 + 3 copies PHP17662
30	581 + 7 copies PHP17662

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

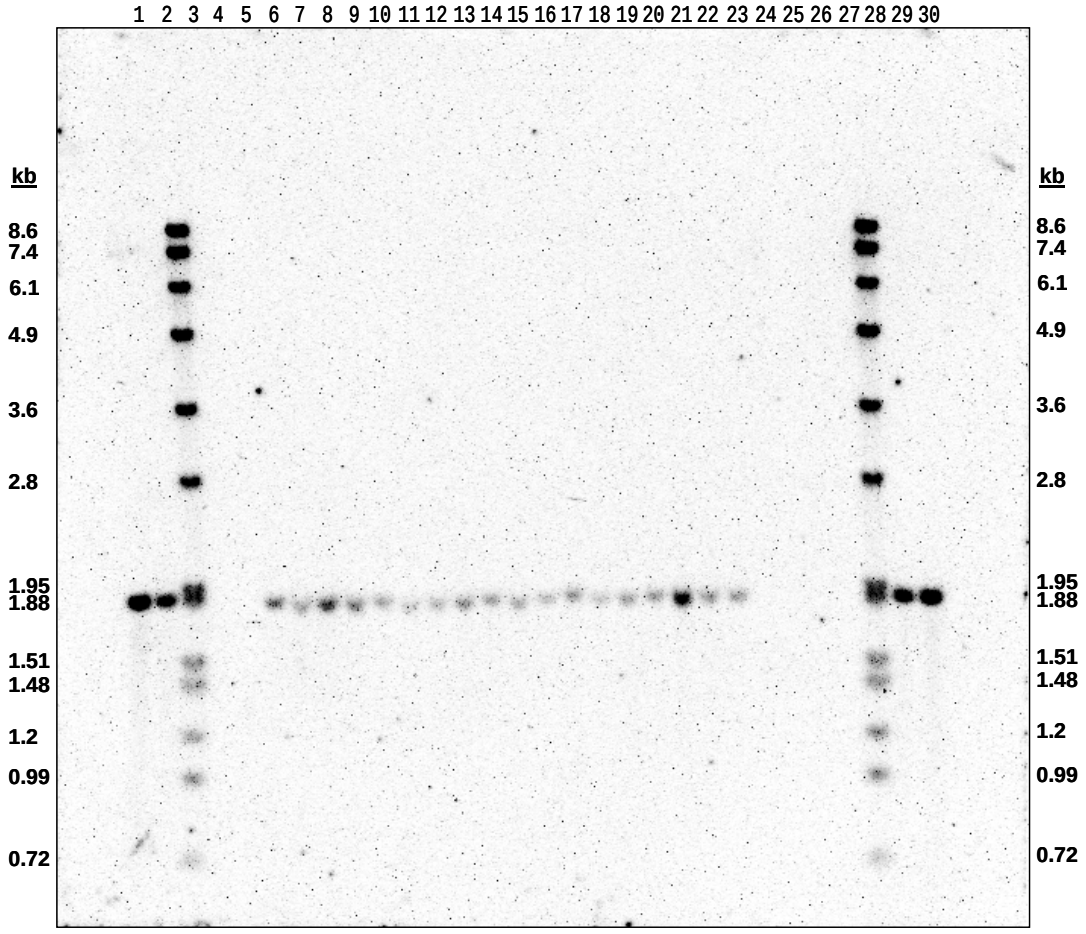


Figure 49. Southern blot analysis of Gel 4; *pat* probe. DNA isolated from transgenic corn event DAS-59122-7 (BC2S1) and Hi-II unmodified corn was digested with *Sac* I and probed with the *pat* gene probe. Approximately 3 μg of genomic DNA was digested and loaded per lane. The gene copy number controls included plasmid PHP17662 at the indicated approximate gene copy number equivalents and 3 μg of either unmodified Hi-II or 581 DNA. Fragment sizes in kb of the DIG-labeled DNA Molecular Weight Marker VII (DIG VII marker) are indicated adjacent to the blot image. **Note:** Approximately 0.5 μg of DAS-59122-7 T66 DNA was loaded in lane 16.

Lane Assignments:

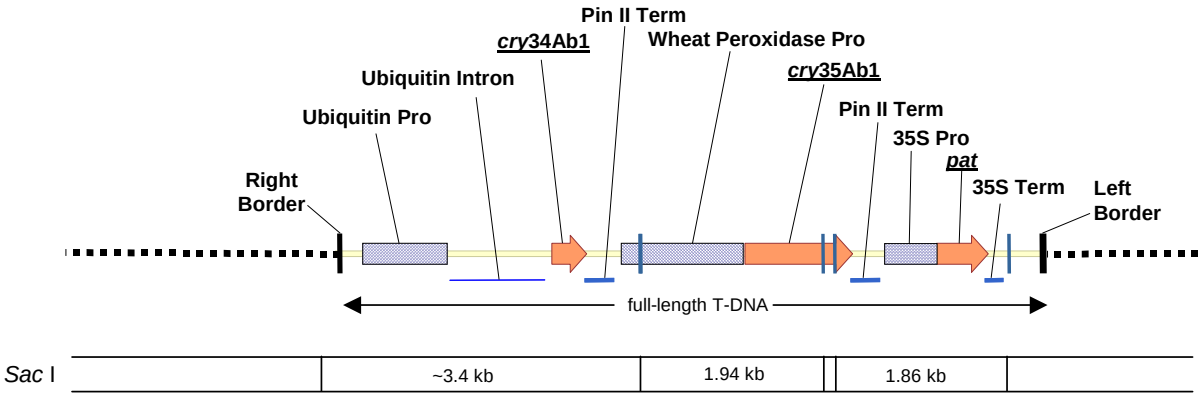
Lane	Sample
1	Hi-II + 7 copies PHP17662
2	Hi-II + 3 copies PHP17662
3	DIG VII
4	Hi-II
5	Blank
6	DAS-59122-7–T56
7	Blank
8	DAS-59122-7–T29 (s)
9	DAS-59122-7–T41 (s)
10	DAS-59122-7–T44 (s)

Lane	Sample
11	DAS-59122-7–T46 (s)
12	DAS-59122-7–T53 (s)
13	DAS-59122-7–T57 (s)
14	DAS-59122-7–T64 (s)
15	DAS-59122-7–T65 (s)
16	DAS-59122-7–T66 (s)
17	DAS-59122-7–T67 (s)
18	DAS-59122-7–T70 (s)
19	DAS-59122-7–T71 (s)
20	DAS-59122-7–T74 (s)

Lane	Sample
21	DAS-59122-7–T75 (s)
22	DAS-59122-7–T11 (s)
23	DAS-59122-7–T22 (s)
24	Blank
25	DAS-59122-7–T78
26	Blank
27	DIG VII
28	581 + 3 copies PHP17662
29	581 + 7 copies PHP17662
30	Blank

(s) indicates a sensitive or null segregant that was negative for expression of both Cry34Ab1 and PAT proteins.

Figure 50. Schematic diagram of DAS-59122-7 T-DNA insertion indicating *Sac* I restriction enzyme sites located in the corn genomic DNA. A putative map of the T-DNA insertion region of DAS-59122-7 based on the Southern analysis conducted. Vertical lines indicate restriction enzyme sites for *Sac* I. The horizontal dotted line represents the plant genomic DNA flanking the insertion. Below the diagram are the fragments that were observed in Southern blot hybridization experiments with the three gene probes, *cry*34Ab1, *cry*35Ab1, and *pat*. Two T-DNA regions are hypothesized to have inserted with T-DNA Right Borders linked and Left Borders forming junctions with the corn genome.



V.C. MENDELIAN INHERITANCE

The Mendelian segregation of the *B.t.* Cry34/35Ab1 corn event DAS-59122-7 was recorded and analyzed, using Chi-square analysis, at eight stages (Table 14, Figure 51). Since DAS-59122-7 should segregate as a single dominate gene, each generation was sprayed with glufosinate-ammonium to eliminate herbicide-susceptible plants to determine if the event was segregating as expected.

.

All plants that were advanced in each breeding generation were tested with Cry34Ab1 immunoassay lateral flow devices (LFD). All of the plants determined to be herbicide tolerant were also found to be positive for Cry34Ab1. In five of the eight generations, no significant deviation from expected segregation ratios was observed (Table 14). Significant deviation from the expected segregation ratio occurred in the BC1, BC4, and BC4S1 generation in only one of two inbreds in each generation. A more consistent pattern of deviations from expected segregation ratios across generations and across inbreds would be anticipated if the event was responsible for these inconsistencies. The most likely explanation for the significant deviations in the BC1 was the smaller sample size. A breeding error that allowed extra susceptible plants in the BC4 and BC4S1 may also explain the deviation seen in the BC1 generation. The deviation in the BC4S1 occurred in only one inbred background and was not seen in either inbred in the BC2S1 generation. Since the majority of the generations showed no significant deviations from the expected ratios, and the deviations that occurred were inconsistent across generations and inbreds, it was concluded that DAS-59122-7 is inherited as a Mendelian dominate gene. A more powerful Chi-square test across all generations for the expected 1:1 ratio

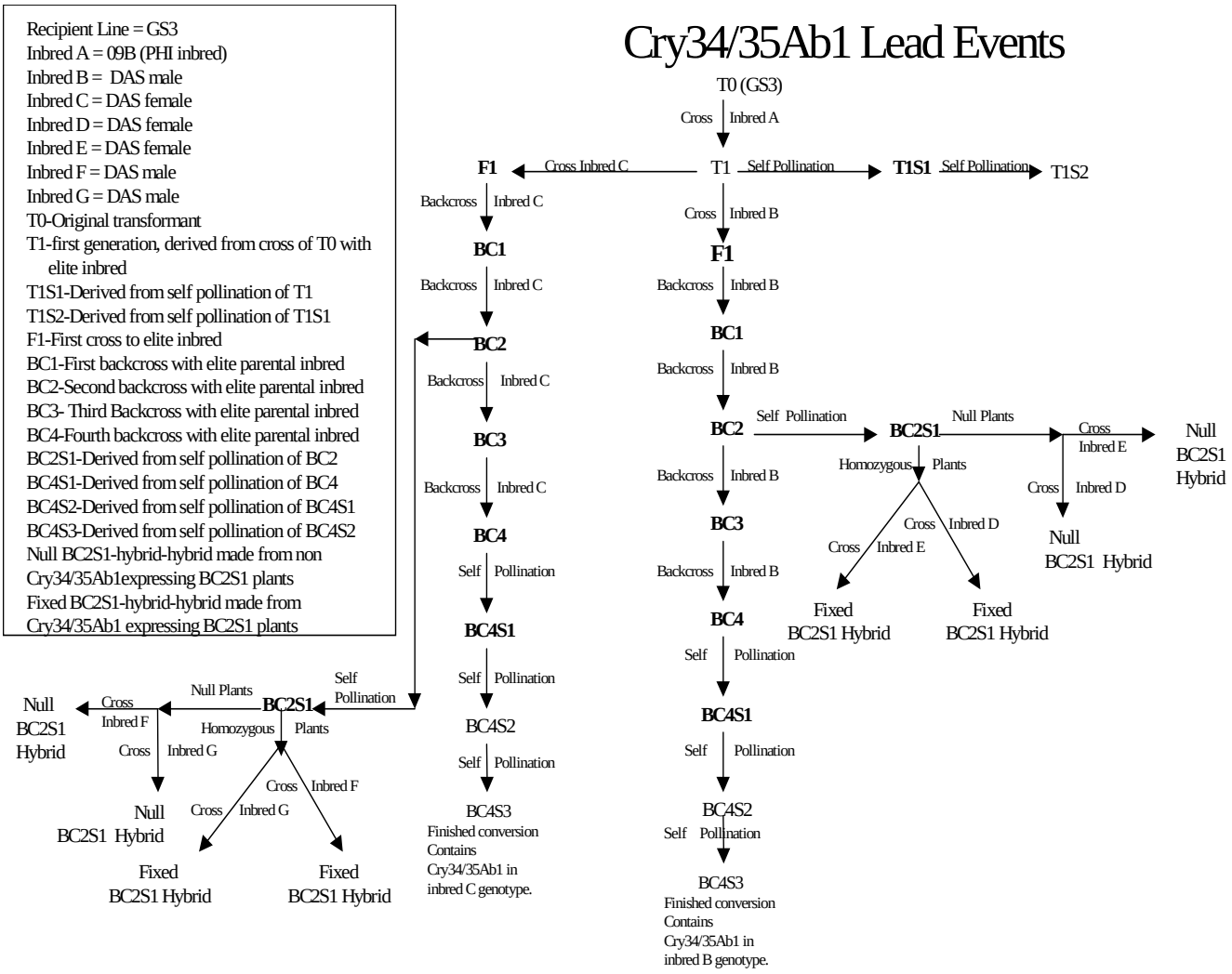
(2644:2750) also results in an insignificant deviation for the expected ratio, as does a test across all generations for an expected ratio of 3:1 (1354:472).

Table 14. Mendelian segregation of *B.t.* Cry34/35Ab1 corn event DAS-59122-7.

Generation	Expected Segregation	Inbred	Number Resistant	Number Susceptible	Chi- Sq Signifigance
T1S1	3:1	Hi-II	34	10	NS @.05
F1	1:1	Inbred B	21	23	NS @.05
	1:1	Inbred C	22	28	NS @.05
BC1	1:1	Inbred B	57	80	P<.05
	1:1	Inbred C	66	78	NS @.05
BC2	1:1	Inbred B	466	466	NS @.05
	1:1	Inbred C	517	471	NS @.05
BC2S1	3:1	Inbred B	267	82	NS @.05
	3:1	Inbred C	302	98	NS @.05
BC3	1:1	Inbred B	431	434	NS @.05
	1:1	Inbred C	415	447	NS @.05
BC4	1:1	Inbred B	451	483	NS @.05
	1:1	Inbred C	198	240	P<.05
BC4S1	3:1	Inbred B	369	121	NS @.05
	3:1	Inbred C	382	161	P<.025

*Data expressed as number of plants expected to be resistant to glufosinate : number of plants expected to be susceptible to glufosinate.
**NS@.05 = non-significant at 0.05: P<.05 or P<.025 = a significant deviation from the expected segregation ratio.

Figure 51. Breeding schematic indicating the generations tested for Mendelian inheritance in Cry34/35Ab1 corn events. Bolded text in the diagram are the generations listed in Table 13.



V.D. CHARACTERISTICS OF INSERTED PROTEINS

Characterization tests were done to confirm the equivalency of the Cry34Ab1 and Cry35Ab1 proteins expressed *in planta* in *B.t.* Cry34/35Ab1 corn line 59122 with the microbially-produced Cry34Ab1 and Cry35Ab1 (via *Pseudomonas fluorescens (Pf))* protein test materials (MR872) used in the non-target organism tests.

Characterization of the biochemical properties of the microbially-produced and plant proteins was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), glycoprotein detection methods, western blot, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and N-terminal sequence analysis. Utilizing these methods the Cry34Ab1 and Cry35Ab1 proteins from *Pf* and transgenic corn (event DAS-59122-7) were shown to be biochemically equivalent. These data support the use of the microbial protein for use in non-target organism testing.

V.D.1. CHACTERISTICS OF THE CRY34AB1 AND CRY35AB1 PROTEINS

B.t. Cry34/35Ab1 event DAS-59122-7 contains two separate parasporal crystal proteins, Cry34Ab1 and Cry35Ab1, with respective molecular weights of 14 kDa and 44 kDa. Both proteins are required together for mortality of western, northern corn rootworm and Mexican corn rootworm larvae (*Diabrotica virgifera virgifera* LeConte, wCRW; *Diabrotica barberi* Smith and Lawrence, nCRW; *Diabrotica virgifera zeae* Kysan and Smith) upon oral ingestion. These insecticidal crystal proteins (ICPs) were derived from the nonmotile *Bacillus thuringiensis* strain PS149B1 (NRRL B-21553). Separate transgenes, *cry*34Ab1 and *cry*35Ab1, optimized for maize expression, were synthesized for the 14 kDa and 44 kDa coding sequences and transformed together into corn plants. The proteins encoded by the synthetic transgenes are identical in sequence to the native *B.t.* crystal proteins.

The maize optimized *cry*34Ab1 transgene encodes a protein comprised of 123 amino acids (Figure 52) and the maize optimized *cry*35Ab1 transgene encodes a protein comprised of 383 amino acids (Figure 53).

Figure 52. Amino acid sequence of the Cry34Ab1 protein

```
1     MSAREVHIDV NNKTGHTLQL EDKTKLDGGR WRTSPTNVAN DQIKTFVAES
51    NGFMTGTEGT IYYSINGEAE ISLYFDNPFA GSNKYDGHSN KSQYEIITQG
101   GSGNQSHVTY TIQTTSSRYG HKS*
```

Figure 53. Amino acid sequence of the Cry35Ab1 protein

```
1     MLDTNKVYEI SNHANGLYAA TYLSLDDSGV SLMNKNDDDI DDYNLKWFLF
51    PIDDDQYIIT SYAANNCKVW NVNNDKINVS TYSSTNSIQK WQIKANGSSY
101   VIQSDNGKVL TAGTGQALGL IRLTDESSNN PNQQWNLTSV QTIQLPQKPI
151   IDTKLKDYPK YSPTGNIDNG TSPQLMGWTL VPCIMVNDPN IDKNTQIKTT
201   PYYILKKYQY WQRAVGSNVA LRPHEKKSYT YEWGTEIDQK TTIINTLGFQ
251   INIDSGMKFD IPEVGGGTDE IKTQLNEELK IEYSHETKIM EKYQEQSEID
301   NPTDQSMNSI GFLTITSLEL YRYNGSEIRI MQIQTSDNDT YNVTSYPNHQ
351   QALLLLTNHS YEEVEEITNI PKSTLKKLKK YYF*
```

The concept of familiarity underpins safety considerations for products of biotechnology (OECD, 1993). The degree to which characteristics of the transgenic trait are well known (that is, familiar) and can be robustly described in the risk assessment influences the confidence that known risk management procedures can assure safety objectives are met (Codex Alimentarius Commission, 2001).

To date, the safety of the plant-expressed Cry proteins has been supported by the experience of decades of safe use of these same proteins in microbial sprays (Narva *et al.*, 2003; Appendix 2, Section 1). The microbial *B.t.* products have never caused any significant adverse human health or environmental effects in more than 40 years of widespread use. In EPA's 1998 Registration Eligibility Decision, the Agency concluded that microbial *B.t.* products pose no unreasonable adverse effects to humans or the environment and that all uses of those products are eligible for re-registration (USEPA, 1998). In addition to this historical experience regarding their safety, there is a general recognition that *B. thuringiensis* and their expressed protein are environmentally ubiquitous. Classification systems describe numerous subspecies of *B.t.* on the basis of flagella antigen serotype (Holt et al., 1993) as well as their cystalline proteins (Crickmore et al., 1998). The distribution of these various subspecies is relatively uniform throughout the world (Martin and Travers, 1989). Although particular isolates of *B.t.* may exhibit differing suites of protein conferring insecticidal activity, they are readily transferable among subspecies through plasmid transmission (USEPA, 1998).

Despite the relatively recent discovery of the unique insecticidal activity of the Cry34/35Ab1 binary protein complex, there is evidence to support familiarity of these proteins (Narva *et al*, 2003; Appendix 2, Section 1). The relationship shown to commerical insectidal proteins, as well as the apparent environmental ubiquity of Cry34/35Ab1 and its homologs, is consistent with other Cry proteins and supports their familiarity despite relatively recent discovery. Genomic serotyping of total genomic DNA for *B.t.* strain collections identified 78 strains containing sequences related to *cry*35Ab1. Crude fermentation broth extracts from a subsample of these strains showed presence of one or both Cry34/35 proteins in 37 of 42 samples. Analysis of nucleic acid and deduced polypeptide sequences reveals that Cry34/35 proteins comprise large families of related ICP. Screening of worldwide collections for homologs to genes coding for Cry34/35Ab1 proteins showed the presence of *cry*34/35 in samples originating from North and South America, and Australasia. The *cry*34/35 genes occurred in samples from fields, stables, parkland, and processing mills and in a variety of matrices (soils, dust, insect bodies, leaf litter). The overall rate of occurrence (1.2%) is comparable to that of a previously approved insect control gene (*cry*3Aa). Further genotyping of six strains positive for *cry*34/35 revealed the presence of other common *Bacillus* insecticidal protein genes (*cry*1, *cry*2, *cry*9, *vip*3), suggesting environmental co-occurrence. Additionally, the Cry35Ab1 protein has homology to binary proteins of *B. sphaericus* that are active against mosquitoes and used in commercial sprays. In conclusion, although the Cry34/35Ab1 proteins do not have a high degree of sequence homology to other Cry proteins currently in commercial transgenic plant products for insect control they are related to commercial microbial products and proteins that are ubiquitous in *B.t.* strains isolated from the environment.

Virtual homology search tools were used to look for structurally related molecules to ascertain possible modes of action since primary sequence homology to other molecules were found to be low. Utilizing PFAM, a large collection of multiple sequence alignments and hidden Markov models covering many common protein domains and

families, two separate domain families in the Cry35Ab1 protein are identified. The largest domain is the Toxin 10 domain common to a family of *Bacillus* insecticidal crystal toxins (Humphreys and Berry, 1998). Strains of *Bacillus* that have this insecticidal activity use a binary toxin comprised of two proteins, P51 and P42. Members of this family are highly conserved between strains of different serotypes and phage groups. Twelve entries of *Bacillus sphaericus* and seven entries of *Bacillus thuringiensis* occupy this family. The second domain found by PFAM in Cry35Ab1 predicted structural homology or similarity to binding portions of diverse molecules containing a carbohydrate binding domain designated $(Q_xW)_3$ (Layton, 2003, Appendix 1). This family contains a diverse collection of molecules including eleven *Bacillus* entries. No structural similarities to the Cry34Ab1 molecule were found using PFAM. However, analysis of the crystalline structure of Cry34Ab1 was found to have structural homology to the beta structure of a pore-forming toxin (equinatoxin II, a pore-forming vertebrate toxin; Dali score of 14) and a sweetener (thaumatin; Dali score of 4) used in foods and confections (unpublished data). However, the extended regions of equinatoxin II, where little structural homology is present to Cry34Ab1, tend to host the most crucial functional domains of the equinatoxin molecule. It is not unexpected nor unusual that the ICP shares putative binding domains and structural homology with other proteins that also have similar binding functions (Layton, 2003, Appendix 1).

Biochemical Characterization of the Microbially-Derived and Plant-Derived Cry34Ab1 and Cry35Ab1 proteins: SDS-PAGE and Western Analyses

SDS-PAGE was performed with crude leaf extracts, immuno-purified Cry34Ab1 and Cry35Ab1 fractions from leaf tissue and microbe-derived Cry34Ab1 and Cry35Ab1 protein from *Pseudomonas fluorescens* (Schafer, *et al.*, 2003; Appendix 2, Section 4). Following electrophoresis, the proteins from two gels electrophoresed simultaneously were transferred to nylon membranes. One membrane was probed with a Cry34Ab1 specific polyclonal rabbit and the other membrane with a Cry35Ab1 specific polyclonal rabbit antibody.

In the toxicology lot preparations of *P. fluorescens*-produced Cry34Ab1 (TSN102172) and Cry35Ab1 (TSN102171), the major protein bands, as visualized on Coomassie stained SDS-PAGE gels, were approximately 14 and 44 kDa respectively. As expected, the corresponding corn-derived Cry34Ab1 and Cry35Ab1 proteins were nearly identical to the microbe-expressed proteins (Figures 54-56). This is consistent with the previous findings for the corn-derived Cry34Ab1 and Cry35Ab1 (Gao, *et al.*, 2000; Appendix 2, Section 41). Predictably, the plant purified fractions contained a minor amount of proteolytic products and impurities in addition to the intact protein. This could be accounted for by the need to highly concentrate the partially purified fractions for visualization on SDS-PAGE.

The microbe-derived Cry34Ab1 and Cry35Ab1proteins each showed a positive signal of the expected size by both SDS-PAGE and western blot analysis (Figures 1-3). This was also the case for the 59.1.22 transgenic corn leaf extract described in this study. The microbe-derived Cry35Ab1, which is susceptible to protease cleavage (Gao *et al*., 2000,

Appendix 2, Section 41), showed a positive signal at both 44 and 40 kDa in western blot and SDS-PAGE analysis. Similarly, the transgenic plant-derived Cry35Ab1 Lot #1 showed a positive signal for both 44- and 40-kDa forms of the protein (Figures 56). In each of the Cry34Ab1 and Cry35Ab1 western blot analyses, no immunoreactive protein was observed in the control sample and no alternate size proteins were seen in the transgenic samples.

Figure 54. SDS-PAGE of event DAS-59122-7 and control 5XH751 corn-leaf extracts and microbe-derived Cry34Ab1 and Cry35Ab1. SDS-PAGE was performed with Bio-Rad Ready gels fitted in a Bio-Rad Ready Gel module. Crude leaf extracts from event DAS-59122-7 and control 5XH751 were mixed 1:1 with Laemmli sample buffer containing 5% freshly added 2-mercaptoethanol and heated for 5 minutes at 100 °C. After a brief centrifugation, 30 µL of the supernatant was loaded directly on the gel. The positive reference standards, microbe-derived Cry34Ab1 (TSN102172) and Cry35Ab1 (TSN102171), were resuspended at 1.0 mg/mL in 20 mM sodium citrate pH 3.5, diluted with PBST and processed as described earlier. Electrophoresis was conducted at a constant amperage of 20 mA per gel for 60 minutes using Bio-Rad Tris/glycine/SDS buffer. After separation the gel was stained with Pierce GelCode Blue protein stain according to the manufacturer's protocol. For clarity not all of the molecular weight markers were labeled. The lanes contained:

Lane 1	BenchMark Molecular Weight Standard – 5 µL
Lane 2	Microbe-Derived Cry34Ab1 (TSN102172) – 3.6 µg
Lane 3	Microbe-Derived Cry35Ab1 (TSN102171) – 3.7 µg
Lane 4	5XH751 Plant #2 Corn-Leaf Extract – 30 µL
Lane 5	DAS-59122-7 Plant #1 Corn-Leaf Extract – 30 µL
Lane 6	MagicMark Molecular Weight Standard – 4 µL
Lane 7	Pierce BlueRanger Molecular Weight Standard – 5 µL

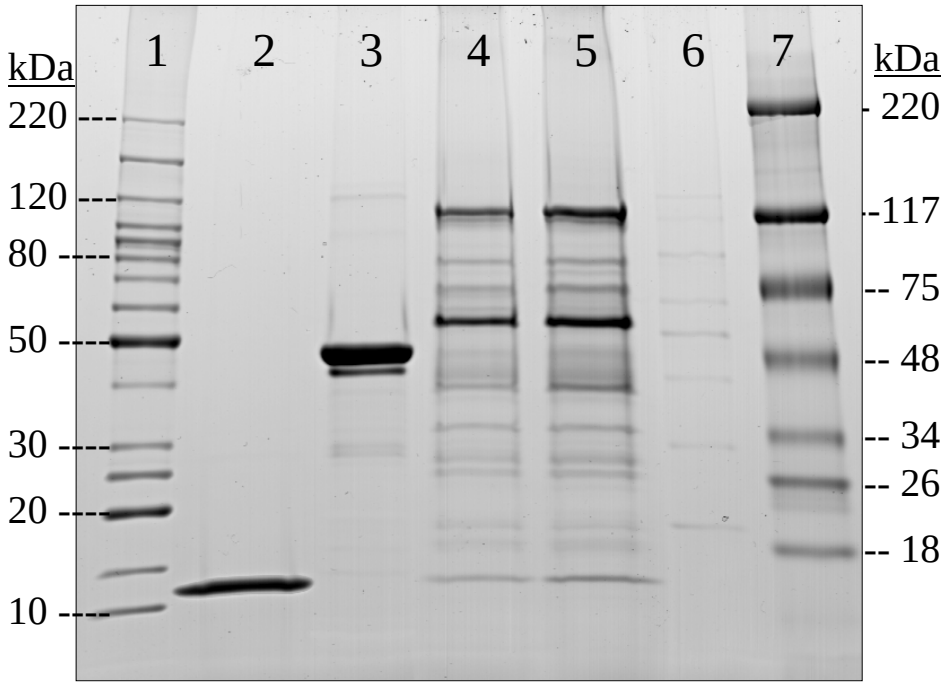


Figure 55. Western blot film of event DAS-59122-7 and control 5XH751 corn-leaf extracts and microbe-derived Cry34Ab1. SDS-PAGE was performed with a Bio-Rad Ready gel fitted into a Bio-Rad Ready Gel module. Crude leaf extracts from event 59.1.22 and control 5XH751 were mixed 1:1 with Laemmli sample buffer containing 5% freshly added 2-mercaptoethanol and heated for 5 minutes at 100 °C. After a brief centrifugation, 30 µL of the supernatant was loaded directly on the gel. The positive reference standard, microbe-derived Cry34Ab1 (TSN102172), was resuspended at 1.0 mg/mL in 20 mM sodium citrate pH 3.5, diluted with PBST and processed as described above. Electrophoresis was conducted at a constant amperage of 20 mA per gel for 60 minutes using Tris/glycine/SDS buffer. After separation, the gel was electro-blotted to a nitrocellulose membrane with a Bio-Rad Ready Gel electrophoretic transfer cell for 2.0 hours at a constant voltage of 50 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. For immunodetection, a Cry34Ab1 specific polyclonal rabbit antibody was used as the primary antibody. A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. Amersham BioSciences chemiluminescent substrate was used for the visualization of the immuno-reactive protein bands when exposed to radiographic film. The BlueRanger molecular weight markers were manually transcribed onto the western blot film after development. For clarity not all of the MagicMark molecular weight markers were labeled. The lanes contained:

Lane 1	MagicMark Molecular Weight Standard – 1.0 µL
Lane 2	Microbe-Derived Cry34Ab1 (TSN102172) – 2.7 ng
Lane 3	5XH751 Plant #2 Corn-Leaf Extract – 30 µL
Lane 4	DAS-59122-7 Plant #1 Corn-Leaf Extract – 30 µL
Lane 5	Pierce BlueRanger Molecular Weight Standard – 5 µL

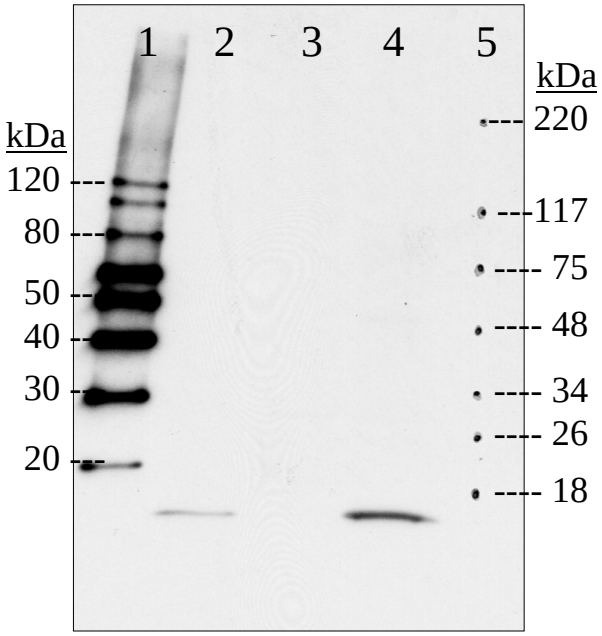
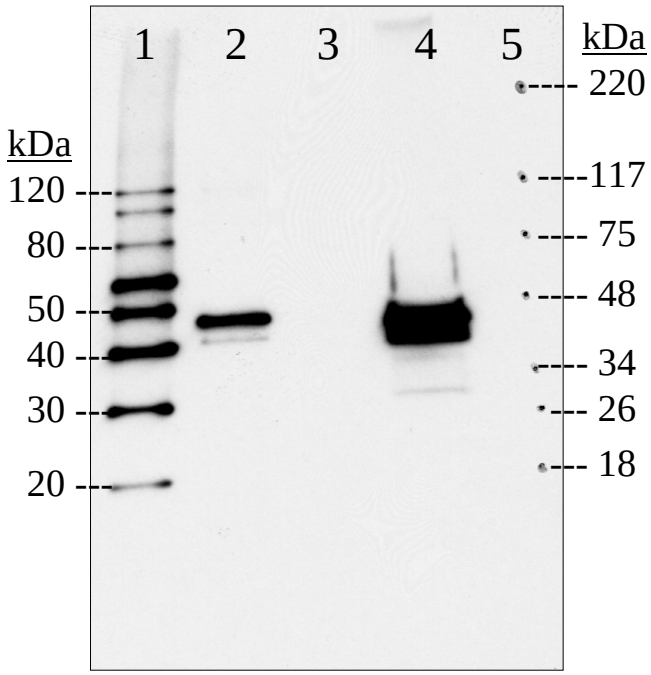


Figure 56. Western blot film of event DAS-59122-7 and control 5XH751 corn-leaf extracts and microbe-derived Cry35Ab1. SDS-PAGE was performed with a Bio-Rad Ready gel fitted into a Bio-Rad Ready Gel module. Crude leaf extracts from event DAS-59122-7 and control 5XH751 were mixed 1:1 with Laemmli sample buffer containing 5% freshly added 2-mercaptoethanol and heated for 5 minutes at 100 °C. After a brief centrifugation, 30 µL of the supernatant was loaded directly on the gel. The positive reference standard, microbe-derived Cry35Ab1 (TSN102171), was resuspended at 1 mg/mL in 20 mM sodium citrate pH 3.5 and diluted with PBST and processed as described above. Electrophoresis was conducted at a constant amperage of 20 mA per gel for 60 minutes using Tris/glycine/SDS buffer. After separation, the gel was electro-blotted to a nitrocellulose membrane with a Bio-Rad Ready Gel electrophoretic transfer cell for 2.0 hours at a constant voltage of 50 volts. The transfer buffer contained 20% methanol and Tris/glycine buffer from Bio-Rad. For immunodetection, a Cry35Ab1 specific polyclonal rabbit antibody was used as the primary antibody. A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary antibody. Amersham BioSciences chemiluminescent substrate was used for the visualization of the immuno-reactive protein bands when exposed to radiographic film. The BlueRanger molecular weight markers were manually transcribed onto the western blot film after development. For clarity not all of the MagicMark molecular weight markers were labeled. The lanes contained:

Lane 1	MagicMark Molecular Weight Standard – 1.0 µL
Lane 2	Microbe-Derived Cry35Ab1 (TSN102172) – 1.9 ng
Lane 3	5XH751 Plant #2 Corn-Leaf Extract – 30 µL
Lane 4	DAS-59122-7 Plant #1 Corn-Leaf Extract – 30 µL
Lane 5	Pierce BlueRanger Molecular Weight Standard – 5 µL



Biochemical Characterization of the Microbially-Derived and Plant-Derived Cry34Ab1 and Cry35Ab1 proteins: Glycosylation Analysis, Tryptic Mass Fingerprinting, N-Terminal Sequencing

Detection of carbohydrates possibly covalently linked to Cry34Ab1 and Cry35Ab1 (microbe- and corn-derived) was assessed by the GelCode Glycoprotein Staining Kit from Pierce (Schafer *et al*, 2003; Appendix 2, Section 4). Microbe-derived and the immunoaffinity-purified Cry34Ab1 and Cry35Ab1 proteins were electrophoresed simultaneously. A glycoprotein, horseradish peroxidase, was loaded as a positive indicator for glycosylation and a non-glycoprotein, soybean trypsin inhibitor, was employed as a negative control. The results showed (Figure 57) that both the corn- and microbe-derived Cry34/35Ab1 proteins had no detectable carbohydrates (Schafer *et al.*, 2003; Appendix 2, Section 4).

The Cry34Ab1 and Cry35Ab1 proteins derived from transgenic corn leaf (event DAS-59122-7) were separated by SDS-PAGE (Figure 58), and the respective bands were excised and subjected to in-gel digestion by trypsin. The resulting peptide mixture was analyzed by MALDI-TOF MS to determine the peptide mass fingerprint coverage. The masses of the detected peptides were compared to those deduced based on potential trypsin cleavage sites in the sequence of corn-derived Cry34Ab1 and Cry35Ab1 proteins. Figures 59 and 60 illustrate the theoretical cleavage which was generated in silico using Protein Analysis Worksheet (PAWS) freeware from Proteometrics LLC. The predicted amino acid digest (and molecular weights) of the corn-derived Cry34Ab1 and Cry35Ab1 proteins is also described in Tables 15 and 16. The Cry34Ab1 and Cry35Ab1 proteins, once denatured, are readily digested by trypsin and will generate numerous peptide peaks.

In the trypsin digest of the transgenic-corn-derived Cry34Ab1 protein, 5 peptides were identified matching the theoretical deduced peptide masses (Table 16). The peptide fragments detected were between residues 5 and 118 of Cry34Ab1. In the trypsin digest of the transgenic-corn-derived Cry35Ab1 protein, 8 peptides were identified matching the theoretical deduced peptide masses (Table 16). The peptide fragments detected were between residues 109 and 329 of Cry35Ab1. The peptide coverage for both the Cry34Ab1 and Cry35Ab1, including the N-terminal sequence data, was very good (54 and 37% respectively). There were several peptides that were not detected in the corn-derived proteins (Tables 15 and 16). This is to be anticipated due to variations of the digestion and ionization of the individual sample preparations and the limitations of the methodology itself. It does not suggest that there is a difference in the corn-derived protein sequence. In addition, there were several unidentified peptides detected in the MALDI-TOF-MS spectrum. Many factors contribute to the formation of these unidentified peptides, such as over digestion (which resulted in non-specific cleavage), self-digestion products of trypsin, and random breakage of peptides during ionization. Results of these analyses indicate that the internal amino acid sequences of both corn-derived Cry34Ab1 and Cry35Ab1 proteins were essentially equivalent to the *P.*

fluorescens-expressed proteins characterized earlier (Gao *et al.*, 2000; Gao and Herman 2000).

Figure 57. SDS-PAGE gel of immuno-purified Cry34/35Ab1 ICP from event DAS-59122-7 corn stained with GelCode Glycoprotein Stain (Panel A) and GelCode Blue Total Protein Stain (Panel B). The immuno-purified, corn-derived Cry34Ab1 and Cry35Ab1 (Lot #1) was concentrated from 250 µL and 750 µL, respectively to ~15 µL and mixed 1:1 with Laemmli buffer. The soybean trypsin inhibitor and horseradish peroxidase were prepared as per the manufacturer's instructions. The proteins were heated at 100 °C for 5 minutes and centrifuged at 20000xg for 1 minute to obtain a clarified supernatant. The resulting mixture was applied directly to a Bio-Rad Ready Gel and the electrophoresis was conducted at a constant amperage of 20 mA for 60 minutes using Bio-Rad Tris/glycine/SDS buffer.. After electrophoresis, the gel was first stained with the GelCode Glycoprotein Staining Kit to visualize glycoproteins. The procedure for glycoprotein staining is briefly described as follows: After electrophoresis, the gel was fixed in 50% methanol for 30 minutes and rinsed with 3% acetic acid. This was followed by an incubation period with the oxidation solution from the staining kit for 15 minutes. The gel was once again rinsed with 3% acetic acid and incubated with Pierce GelCode glycoprotein staining reagent for 15 minutes. Finally, the gel was immersed in the reduction solution for 5 minutes, and then rinsed with 3% acetic acid. The glycoproteins (with a detection limit of 0.625 ng per band) were visualized as magenta bands on a light pink background. After the glycoprotein staining was complete, the gel was scanned with a Hewlett Packard desktop scanner to obtain a permanent visual record of the gel. Next, to visualize the total protein contained in the gel, the gel was stained with Pierce GelCode Blue according to the manufacturer's protocol. Finally, the gel was scanned with a Molecular Dynamics densitometer to obtain a permanent visual record of the gel. For clarity not all of the BenchMark molecular weight markers were labeled in Panel B. The BenchMark molecular weight markers are not glycoproteins and were therefore not visualized in the gel stained with the glycoprotein stain (Panel A). The lanes contained:

Lane 1	BlueRanger Molecular Weight Standard – 5 µL
Lane 2	Soybean Trypsin Inhibitor – 10 µg
Lane 3	Soybean Trypsin Inhibitor – 5.0 µg
Lane 4	Soybean Trypsin Inhibitor – 2.0 µg
Lane 5	Immuno-purified, corn-derived Cry35Ab1 from event DAS-59122-7 – 30 µL
Lane 6	Immuno-purified, corn-derived Cry34Ab1 from event DAS-59122-7 – 30 µL
Lane 7	Horseradish Peroxidase – 2.0 µg
Lane 8	Horseradish Peroxidase – 5.0 µg
Lane 9	Horseradish Peroxidase – 10 µg
Lane 10	BenchMark Molecular Weight Standard – 5 mL

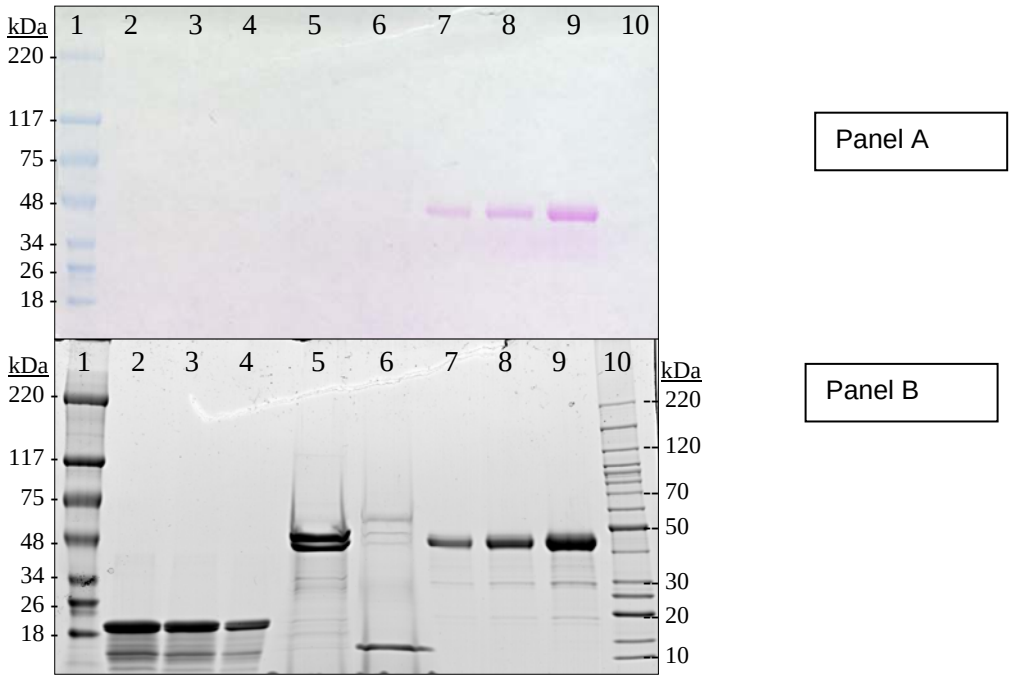


Figure 58. SDS-PAGE gel of corn-derived Cry34Ab1 and Cry35Ab1 (event DAS-59122-7) proteins for MALDI-TOF peptide mass fingerprinting. Cry34Ab1 and Cry35Ab1 Lot #2 was concentrated to ~1000 µL and 25 µL, respectively, and 20 µL of each sample was removed for MALDI-TOF peptide mass fingerprint analysis. The concentrated proteins were mixed with 10 µL of Laemmli sample buffer and heated for 5 minutes at 100 °C. After a brief centrifugation, the supernatants were loaded directly on the gel. SDS-PAGE was performed with a Bio-Rad Ready gel fitted in a Ready Gel module. After separation the gel was stained with Pierce GelCode Blue protein stain according to the manufacturer's protocol. Finally, the gel was scanned with a Molecular Dynamics densitometer to obtain a permanent visual record of the gel. The respective Cry34Ab1 and Cry35Ab1 bands were excised from the gel, placed into siliconized Eppendorf microcentrifuge tubes, and destained with 50% acetonitrile in 25 mM NH_4HCO_3. The gel pieces were dried using vacuum centrifugation, and digested with sequencing grade trypsin overnight (approximately 17 hours) at 37 °C. The peptides were extracted with 50% acetonitrile in 0.5% TFA. After brief centrifugation to pellet the gel pieces, the supernatant containing the peptides was decanted and dried in a Savant Speed-Vac and the samples were stored at –20 °C until MALDI-TOF MS analysis. For clarity not all of the molecular weight markers were labeled. The lanes contained:

Lane 1	BenchMark Molecular Weight Standard – 5 µL
Lane 2	Immuno-purified Cry34Ab1 (from event DAS-59122-7) – 30 µL
Lane 3	Immuno-purified Cry35Ab1 (from event DAS-59122-7) – 30 µL

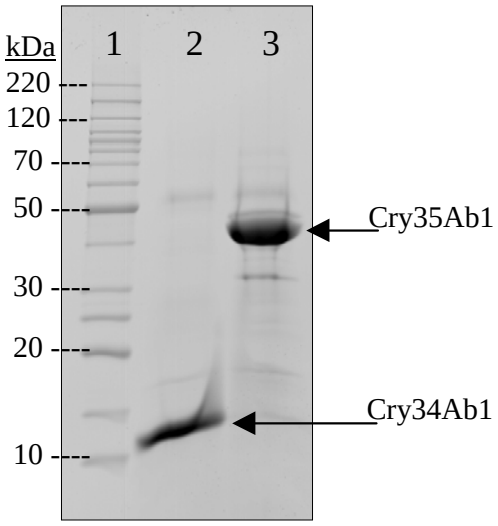


Figure 59. Expected amino acid sequence and peptide fragments of corn-derived Cry35Ab1. Molecular weight: 43.8 kDa. Trypsin Cleavage (at K and R) of corn-derived Cry35Ab1. Alternating blocks of upper and lower case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion. The numbers on the left and right sides indicate the amino acid residue numbers.

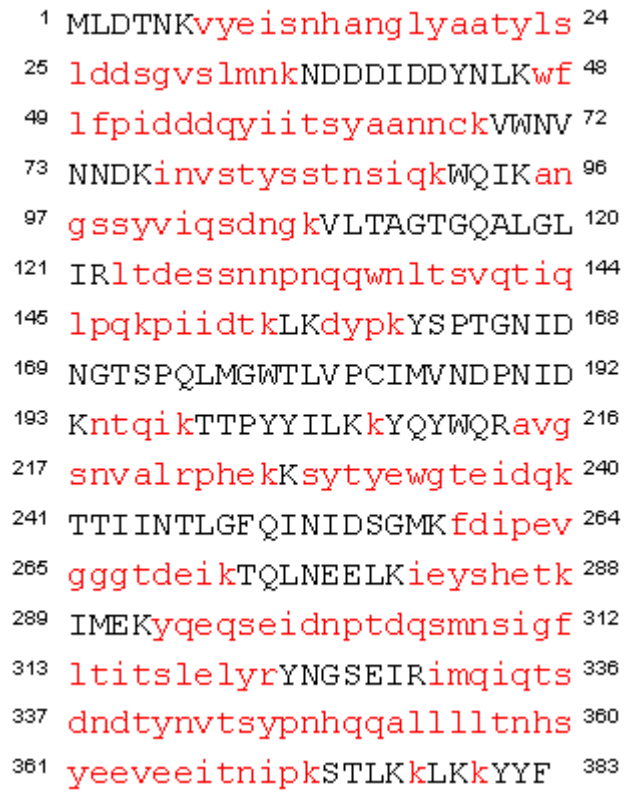


Figure 60. Expected amino acid sequence and peptide fragments of corn-derived Cry34Ab1. Molecular weight: 13.6 kDa. Trypsin Cleavage (at K and R) of corn-derived Cry34Ab1. Alternating blocks of upper and lower case letters within the amino acid sequence are used to differentiate the potential peptides after trypsin digestion.

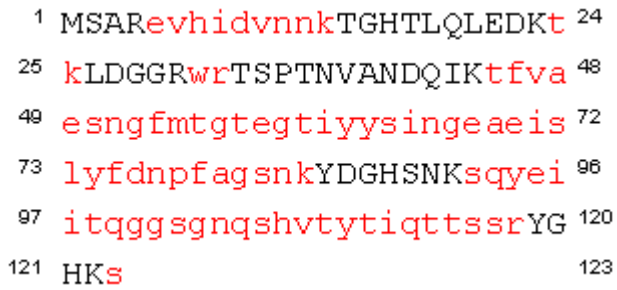


Figure 11. Peptide mass fingerprint coverage of the immuno-affinity purified Cry35Ab1 protein from DAS-59122-7 leaf. Shaded letters represent peptide fragments detected by MALDI-TOF MS. The bolded letters indicate the peptide sequence confirmed by N-terminal sequencing. The amount of coverage was 140 amino acid residues out of 383 or 37%.

```
1   MLDTNKVYEISNHANGLYAA 20
21  TYLSLDDSGVSLMNKNDDDI 40
41  DDYNLKWFLFPIDDDQYIIT 60
61  SYAANNCKVWNVNNDKINVS 80
81  TYSSTNSIQKWQIKANGSSY 100
101 VIQSDNGKVLTAGTGQALGL 120
121 IRLTDESSNNPNQQWNLTSV 140
141 QTIQLPQKPIIDTKLKDYPK 160
161 YSPTGNIDNGTSPQLMGWTL 180
181 VPCIMVNDPNIDKNTQIKTT 200
201 PYYILKKYQYWQRAVGSNVA 220
221 LRPHEKKSYTYEWGTEIDQK 240
241 TTIINTLGFQINIDSGMKFD 260
261 IPEVGGGTDEIKTQLNEELK 280
281 IEYSHETKIMEKYQEQSEID 300
301 NPTDQSMNSIGFLTITSLEL 320
321 YRYNGSEIRIMQIQTSDNDT 340
341 YNVTSYPNHQQALLLLTNHS 360
361 YEEVEEITNIPKSTLKKLKK 380
381 YYF                 383
```

Figure 62. Peptide mass fingerprint coverage of the immuno-affinity purified Cry34Ab1 protein from DAS-59122-7 leaf. Shaded letters represent peptide fragments detected by MALDI-TOF MS. The bolded letters indicate the peptide sequence confirmed by N-terminal sequencing. Amount of coverage was 66 amino acid residues out of 123 or 54%.

```
1   MSAREVHIDVNNKTGHTLQL 20

21  EDKTKLDGGRWRTSPTNVAN 40
41  DQIKTFVAESNGFMTGTEGT 60
61  IYYSINGEAEISLYFDNPFA 80
81  GSNKYDGHSNKSQYEIITQG 100
101 GSGNQSHVTYTIQTTSSRYG 120
121 HKS                  123
```

Table 15. Tryptic peptide mass data (m/z $[M+H]^+$) of corn-derived Cry34Ab1 protein obtained by MALDI-TOF mass spectroscopy.

Cry34 residue number	Theoretical mass[a] (*m/z*)	DAS-59122-7 corn Cry34Ab1 [M+H]
1-4	463.56	NTS[b]
5-13	1067.16	1067.46
14-23	1141.24	1141.47
24-25	247.29	ND[c]
26-30	516.55	517.20
31-32	360.42	ND
33-44	1287.39	1287.55
45-84	4343.69	ND
85-91	819.83	ND
92-118	2944.11	2943.34
119-122	503.56	ND
123-123	105.09	ND

Notes:

[a] Two digit decimals were used for mass data in this table although raw data obtained from the MALDI-TOF-MS spectrometer were shown in 4 digit decimals. A peptide was considered a match if its *m/z* is within +/- 1.0 error range of its theoretical *m/z*.

[b] NTS: Peptide fragment detected by N-terminal sequencing and was shown to be missing residue #1.

[c] ND: Peptide fragment not detected.

Table 16. Tryptic peptide mass data (m/z $[M+H]^+$) of corn-derived Cry35Ab1 protein obtained by MALDI-TOF mass spectroscopy.

Cry35Ab1 residue number	Theoretical mass[a] (*m/z*)	DAS-59122-7 corn Cry35Ab1 $[M+H]^+$
1-6	720.84	NTS[b]
7-35	3146.47	ND[c]
36-46	1339.33	ND
47-68	2637.94	ND
69-76	988.07	ND
77-90	1541.68	ND
91-94	573.69	ND
95-108	1439.50	ND
109-122	1369.62	1369.87
123-154	3652.02	3651.17
155-156	259.35	ND
157-160	521.57	ND
161-193	3592.07	ND
194-198	602.69	ND
199-206	998.18	ND
207-207	146.19	ND
208-213	943.03	943.47
214-226	1377.56	1377.81
227-227	146.19	ND
228-240	1619.70	1619.80
241-258	1966.28	ND
259-272	1476.60	1476.78
273-280	974.08	ND
281-288	1006.08	ND
289-292	519.66	ND
293-322	3493.80	3493.89
323-329	837.89	838.43
330-372	5006.48	ND
373-376	447.53	ND
377-377	146.19	ND
378-379	259.35	ND
380-380	146.19	ND
381-383	491.54	ND

Notes:

[a] Two digit decimals were used for mass data in this table although raw data obtained from the MALDI-TOF-MS spectrometer were shown in 4 digit decimals. A peptide was considered a match if its *m/z* is within +/- 1.0 error range of its theoretical *m/z*.

[b] NTS: Peptide fragment detected by N-terminal sequencing and was shown to be intact.

[c] ND: Peptide fragment not detected.

The amino acid residues at the N-termini of the corn-derived Cry34Ab1 and Cry35Ab1 proteins (immuno-affinity purified from corn event DAS-59122-7) were sequenced and compared to the sequence of the microbe-derived proteins (Schafer *et al.*, 2003; Appendix 2, Section 4; Gao *et al.*, 2000 and Schmidt, 2003). The Edman degradation reaction was performed on the first 10 residues and the amino acid sequences were obtained and the results are summarized in Table 17. The N-terminus of the transgenic corn-derived Cry35Ab1 was sequenced and it was determined that the residues were

identical to those predicted by the gene sequence and those determined by Gao et al. (Table 17, rows 4-6). For the transgenic corn-derived Cry34Ab1 protein, the first amino acid on the N-terminus (methionine) was missing and the subsequent ten amino acids sequenced matched residues 2 through 11 of the expected protein sequence (Table 17, rows 1-3). This result suggested that after translation in plants, the N-terminal methionine was cleaved by a protease. This result was similar to the findings of Gao and co-workers for transgenic corn-derived Cry34Ab1 (event TC5638) and Schmidt for the *P. fluorescens* expressed Cry34Ab1 standard protein. When the *P. fluorescens*- and transgenic corn-derived Cry34Ab1 were sequenced, it was determined that the N-terminal methionine was removed from the N-terminus in both organisms. This result is not uncommon as it is known that the N-terminus can be cleaved by aminopeptidases in both prokaryotic and eukaryotic systems (Li and Chang, 1995).

Table 17. N-terminal sequence of Cry34Ab1 and Cry35Ab1 proteins from corn event DAS-59122-7.

1) Cry34Ab1	M^{1}	S	A	R	E	V	H	I	D	V	N^{11}
2) Corn Cry34Ab1[a]	$-^{1}$	S	A	R	E	V	H	I	D	V	N^{11}
3) *P.f.* Cry34Ab1	$-^{1}$	S	A	R	E	V	H	I	D	V	N^{11}
4) Cry35Ab1	M^{1}	L	D	T	N	K	V	Y	E	I^{10}	
5) Corn Cry35Ab1[a]	M^{1}	L	D	T	N	K	V	Y	E	I^{10}	
6) P.f. Cry35Ab1	M^{1}	L	D	T	N	K	V	Y	E	I^{10}	

1: Expected N-terminal sequence of the first 11 amino acid residues of Cry34Ab1

2: Detected N-terminal sequence of the immuno-affinity purified, corn-derived Cry34Ab1[a].

3. Detected N-terminal sequence from *P. fluorescens*-derived Cry34Ab1 (Schmidt, 2003)

4: Expected N-terminal sequence of the first 10 amino acid residues of Cry35Ab1.

5: Detected N-terminal sequence of the immuno-affinity purified, corn-derived Cry35Ab1[a].

6: Detected N-terminal sequence from *P. fluorescens*-derived Cry35Ab1 (Gao *et al.*, 2000)

Notes: Numbers in superscript indicate amino acid residue numbers in the sequence.
[a]: Cry34Ab1 and Cry35Ab1 were purified from event DAS-59122-7.

Amino acid residues:
C: cysteine
D: aspartic acid
E: glutamic acid
G: glycine
I: isoleucine
L: leucine
M: methionine
N: asparagine
P: proline
Q: glutamine
R: arginine
S: serine
T: threonine
V: valine
Y: tyrosine
-: missing amino acid residue

V.D.2. CHARACTERISTICS OF THE PAT PROTEIN

The phosphinothricin acetyltransferase (PAT) protein acetylates phosphinothricin, or its precursor demethylphosphinothricin, conferring tolerance to a chemically synthesized phosphinothricin such as the herbicide glufosinate-ammonium. Acetylation converts phosphinothricin to an inactive form that is no longer toxic to corn plants. Glufosinate-ammonium is a broad spectrum, non-systemic, non-selective herbicide. Individual corn plants tolerant to glufosinate-ammonium herbicide can be readily identified in the field through either spray application or application of the herbicide to leaves. Tolerance to the herbicide provides improved weed control management. The PAT enzyme catalyzes the conversion of L-phosphinothricin, the active ingredient in glufosinate-ammonium, to an inactive form and thereby detoxifies the glufosinate-ammonium herbicide.

The plant-optimized *pat* transgene encodes a protein of 183 amino acids (Figure 63).

Figure 63. Amino Acid Sequence of the PAT Protein

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

Characterization of the biochemical properties of the microbially-produced and plant-produced PAT proteins was accomplished through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), glycoprotein detection methods, western blot, matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and N-terminal sequence analysis (Schafer and Collins, 2003; Appendix 2, Section 5). Utilizing these methods, the PAT protein from the microbial preparation and the transgenic corn (event DAS-59122-7) were shown to be biochemically equivalent.

Biochemical Characterization of the Microbially-Derived and Plant-Derived PAT Protein: SDS PAGE and Western Analyses

Biochemical analyses were performed to characterize the PAT protein in both *E. coli*-produced and transgenic leaf extracts from greenhouse-grown corn plants (Schafer and Collins, 2003; Appendix 2, Section 5). The analyses included sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blotting and enzyme linked immunosorbent assay (ELISA). SDS-PAGE was used to determine if the microbial PAT protein was of the expected molecular weight. Both western blotting and ELISA analysis was used to determine if both of the test materials contained protein immunoreactive to antibodies specific to the PAT protein. In addition, western blot was used to investigate if the microbe- and transgenic plant- derived proteins were at the expected molecular weight.

Analysis of the PAT microbial protein by SDS-PAGE demonstrated that the protein present in the sample produced a band at a molecular weight of ~23 kDa (Figure 64). This result was as expected since the authenticity of the microbe-derived PAT was shown by MALDI-TOF (matrix-assisted laser desorption/ionization-time of flight mass spectrometry) peptide mass fingerprinting in a previous study (Korjagin 2000). In addition, the microbe-derived PAT protein and all five leaf extracts of DAS-59122.7 showed a positive signal of expected size by western blot analysis (Figure 65). The immunoreactive signal of each plant extract was very similar. In addition, no immunoreactive protein was observed in the control samples, and no alternate size proteins were seen in the transgenic or microbial samples as shown by ELISA and western blot analysis respectively.

Figure 64. SDS-PAGE of microbe-derived PAT, DAS-59122-7 and 5XH751 corn leaf extracts stained with GelCode Blue Protein Stain. For SDS-PAGE analysis, 50 µL of the plant extracts from DAS-59122-7 (plant #'s: 1, 3, 4, 7 and 8) and 5XH751 (plant #: 2) was mixed with 50 µL of freshly prepared Laemmli buffer containing 5% 2-mercaptoethanol and heated for 5 minutes at 100 °C. The supernatant was collected by centrifugation for 1 minute at 20,000xg. For the reference protein, 10 µL of the microbe-derived PAT (TSN101850, a.i.: 620 µg/mL) was diluted with 30 µL Laemmli buffer and processed as described for the plant extracts. The resulting soluble protein fractions were applied directly to the SDS-PAGE gel for total protein visualization. SDS-PAGE was performed with a 4-20% Bio-Rad Ready gel fitted into a Bio-Rad Ready Gel module. The microbe-derived PAT was loaded at 3.1 g/well (20 µL), and the crude corn extracts were loaded at 30 µL/well. The electrophoresis was conducted at a constant amperage of 25 mA for ~70 minutes using Tris/glycine/SDS buffer. After separation, the gel was washed extensively with water and stained with GelCode Blue stain to visualize the total protein. The lanes contained:

Lane 1	BenchMark Molecular Weight Standards – 5 µL
Lane 2	DAS-59122-7 – Plant # 1 – 30 µL
Lane 3	DAS-59122-7 – Plant # 3 – 30 µL
Lane 4	DAS-59122-7 – Plant # 4 – 30 µL
Lane 5	DAS-59122-7 – Plant # 7 – 30 µL
Lane 6	DAS-59122-7 – Plant # 8 – 30 µL
Lane 7	Blank
Lane 8	5XH751 – Plant # 2 – 30 µL
Lane 9	Microbe-Derived PAT – 3.1 µg
Lane 10	MagicMark Molecular Weight Standards – 4 µL

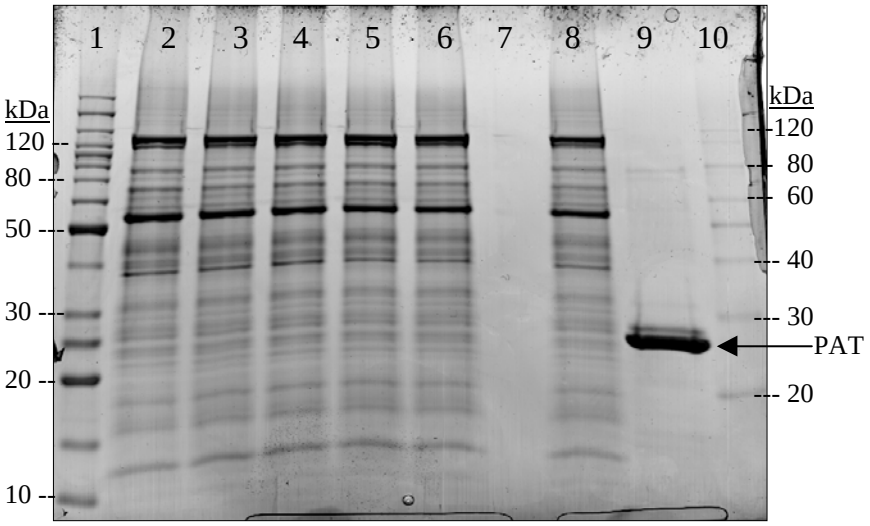
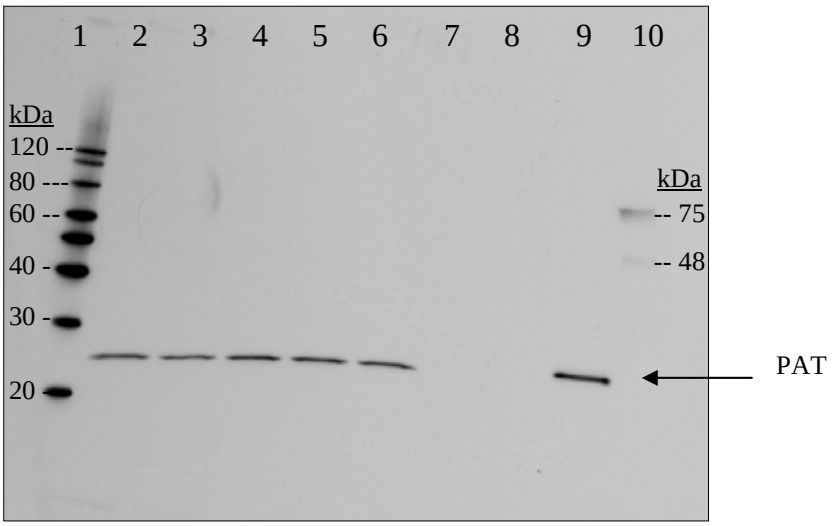


Figure 65. Anti-PAT protein western blot film of microbe-derived PAT, DAS-59122-7 and 5XH751 maize leaf extracts. For western blot analysis, 50 µL of plant extracts from DAS-59122-7 (plant #'s: 1, 3, 4, 7 and 8) and 5XH751 (plant #: 2) was mixed with 50 µL of freshly prepared Laemmli buffer containing 5% 2-mercaptoethanol and heated for 5 minutes at 100 °C. The supernatant was collected by centrifugation for 1 minute at 20,000xg. The extracts were loaded at 30 µL/well. The microbe-derived PAT (TSN101850, a.i.: 620 µg/mL) was diluted 1:1000 in PBST (0.62 ng/µL), mixed 1:1 with freshly prepared Laemmli buffer, and heated at 100 °C for 5 minutes. The supernatant was collected by centrifugation for 1 minute at 20,000xg and the PAT protein was loaded at 3.1 ng/well (10 µL). The electrophoresis was conducted utilizing a 4-20% Bio-Rad Ready gel fitted into a Bio-Rad Ready Gel module at a constant amperage of 25 mA for ~70 minutes using Tris/glycine/SDS buffer. After separation the gel was electro-blotted to a nitrocellulose membrane with a Bio-Rad Ready Gel-Blotting electrophoretic transfer cell for 120 minutes under a constant voltage of 50 volts. For immunodetection, Anti-PAT specific polyclonal rabbit antibodies (Lot #: 69:74A, EnviroLogix Portland, Maine) were used as the primary antibody. A conjugate of goat anti-rabbit IgG (H+L) and horseradish peroxidase was used as the secondary/detection antibody. Amersham BioSciences chemiluminescent substrate was used for development and visualization of the immunoreactive protein bands. The membrane was exposed to Pierce CL-X Posure film for various time points and subsequently developed with a Konica SR-X film developer. The lanes contained:

Lane 1	MagicMark Molecular Weight Standards – 1 µL
Lane 2	DAS-59122-7 – Plant # 1 – 30 µL
Lane 3	DAS-59122-7 – Plant # 3 – 30 µL
Lane 4	DAS-59122-7 – Plant # 4 – 30 µL
Lane 5	DAS-59122-7 – Plant # 7 – 30 µL
Lane 6	DAS-59122-7 – Plant # 8 – 30 µL
Lane 7	Blank
Lane 8	5XH751 – Plant # 2 – 30 µL
Lane 9	Microbe-Derived PAT – 3.1 ng
Lane 10	BlueRanger Prestained Peroxidase Labeled Standard – 5 µL



V.D.3. CRY34AB1 AND CRY35AB1 EXPRESSION IN PLANT TISSUES

A field expression study was conducted in Chile in 2002-2003 which contained six (6) separate field trials in which the genetically modified hybrid line 59122 and its near isoline control EE05F/GR581 (also known as 91) were grown. Expression levels of theCry34Ab1, Cry35Ab1 and PAT proteins were determined from tissues at various growth stages (Tables 17 and 18) from the transgenic hybrid line and the appropriate control line.

Table 17. Definitions of physiological time-points in corn.

Time-Point	Definitions of Time-Points
V9	The growth stage when the collar of the fourth (4^{th}) leaf becomes visible.
R1	The growth stage when silks become visible.
R4	The growth stage when the material within the kernel produces a doughy consistency. This stage can occur as early as 24 days after pollination.
Maturity	The typical harvest maturity for grain.
R6	Maturity, the typical harvest maturity for grain.

Table 18. List of corn tissues collected, growth stage, sample size, and number of samples collected per entry.

Block	Tissue	Growth Stage	Sample Size	No. Samples / Block	
				Control	Test
1 (expression)	Leaf	V9	1 leaf	1	5
	Whole plant	V9	3 plants[1]	1	1
	Root	V9	1 plant	1	3
	Pollen	R1	1 plant	1	5
	Stalk	R1	1 plant	1	5
	Root	R1	1 plant	1	5
	Leaf	R1	1 leaf	1	5
	Whole plant	R1	3 plants[1]	1	1
	Forage	R4	3 plants[1]	1	1
	Root	R4	1 plant	1	3
	Leaf	R4	1 leaf	1	3
	Grain	Maturity	1 ear	1	5
	Whole plant	R6	3 plants[1]	1	1
	Root	R6	1 plant	1	3
	Leaf	R6	1 leaf	1	3
2 – 4 (nutrient composition)	Forage	R4	3 plants[1]	1	1
	Grain	Maturity	1 ear	5	5

[1]Three plants combined (pooled) to make one sample

The experimental design included six (6) field sites. Each field site (site codes: BU01, BU02, LI01, LI02, VI01, and VI02) employed a randomized complete block design containing four blocks (replicates) with test and control substances (seeds) planted in 2-row plots located randomly within each block, bordered by 12 rows of non-transgenic corn. An alley distance of at least 0.9 meters separated each block. A non-transgenic corn line of similar relative maturity as the transgenic corn plants bordered each 2-row plot. At each location block 1 was designated for collection of samples for protein determination. Blocks 2, 3, and 4 were designated for the collection of samples for nutrient composition analysis.

Whole plant and forage samples were dried at the field site to remove all moisture. All other samples were lyophilized in frozen conditions under vacuum to remove all moisture. The lyophilization time varied depending on the sample size, sample composition, and amount of water in the sample. Between lyophilization and grinding, samples were stored frozen.

Processed corn tissues were weighed into 1.1 ml tubes at the following target weights: 5 mg for pollen, 10 mg for leaf, 20 mg for grain and root, and 30 mg for stalk and whole plant tissues. Each sample was extracted with 600 µl of phosphate buffered saline solution containing 0.05% Tween 20 (PBST) using a Spex Certiprep GenoGrinder. Following centrifugation, the resultant extract supernatants were diluted and analyzed for Cry34Ab1, Cry35Ab1 and PAT amounts using a specific Enzyme Linked ImmunoSorbent Assay (ELISA) for each protein.

The Cry34Ab1 ELISA Kit produced by Beacon Analytical Systems, Inc., utilizes a sequential “sandwich” format for the quantification of Cry34Ab1 in plant tissue extracts. Briefly, standards (analyzed in triplicate wells) and diluted tissue extracts (analyzed in duplicate wells) were incubated in a 96-well stabilized ELISA plate that was pre-coated with a Cry34Ab1-specific antibody. Unbound substances were washed from the plate and a different Cry34Ab1-specific antibody that was conjugated to the enzyme horseradish peroxidase (HRP) was added to each well. Bound Cry34Ab1 protein was sandwiched between the antibody coated on the plate and the antibody-HRP conjugate. At the end of the incubation, unbound substances were washed from the plate. Detection of the bound Cry34Ab1-antibody complex was accomplished by the addition of a substrate solution, which generated a colored product in the presence of HRP. The reaction was stopped with stop solution (Hydrochloric acid) and the optical density of each well was determined using a Molecular Devices plate reader with a wavelength setting of 450 nm minus 650 nm. SOFTmax® PRO software performed the calculations that generated the quadratic fit for the standard curve and converted the sample OD values to Cry34Ab1 protein concentration values. The mean concentration from the duplicate wells in ng/ml was used in the calculation of the concentration of the sample in ng/mg dry weight.

The Cry35Ab1 ELISA Kit produced by Beacon Analytical Systems, Inc., also utilizes a sequential “sandwich” format for the quantification of Cry35Ab1 in plant tissue extracts.

Briefly, standards (analyzed in triplicate wells) and diluted tissue extracts (analyzed in duplicate wells) were incubated in a 96-well stabilized ELISA plate that was pre-coated with a Cry35Ab1-specific antibody. Unbound substances were washed from the plate and a different Cry35Ab1-specific antibody that was conjugated to the enzyme horseradish peroxidase (HRP) was added to each well. Bound Cry35Ab1 protein was sandwiched between the antibody coated on the plate and the antibody-HRP conjugate. At the end of the incubation, unbound substances were washed from the plate. Detection of the bound Cry35Ab1-antibody complex was accomplished by the addition of a substrate solution, which generated a colored product in the presence of HRP. The reaction was stopped with stop solution (Hydrochloric acid) and the optical density of each well was determined using a Molecular Devices plate reader with a wavelength setting of 450 nm minus 650 nm. SOFTmax® PRO software performed the calculations that generated the quadratic fit for the standard curve and converted the sample OD values to Cry35Ab1 protein concentration values. The mean concentration from the duplicate wells in ng/ml was used in the calculation of the concentration of the sample in ng/mg dry weight.

Neither Cry34Ab1 nor Cry35Ab1 were detected in any tissues of the control line 91 (Tables 19 - 20).

Table 19. Summary of expression levels of Cry34Ab1 protein (ng/mg Tissue Dry Weight) measured in tissues collected from corn hybrid control line 91.

Growth Stage	Tissue	Event	Mean of Cry34Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of Cry34Ab1 (ng/mg Tissue Dry Weight) [1]	Number of Samples[2]
V9	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	5 / 5
R1	Leaf	91	0	0	0 - 0	6 / 6
	Pollen	91	0	0	0 - 0	6 / 6
	Stalk	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	6 / 6
R4	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Forage	91	0	0	0 - 0	6 / 6
Maturity	Grain	91	0	0	0 - 0	6 / 6
R6	Leaf	91	0	0	0 - 0	5 / 5
	Root	91	0	0	0 - 0	5 / 5
	Whole Plant	91	0	0	0 - 0	5 / 5

[1] Sample LOQ for Cry34Ab1: 0.18 ng/mg dry weight for leaf, 0.36 ng/mg dry weight for pollen, 0.12 ng/mg dry weight for stalk, 0.09 ng/mg dry weight for root, and 0.072 ng/mg dry weight for grain and whole plant tissues.

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

Table 20. Summary of expression levels of Cry35Ab1 Protein (ng/mg tissue dry weight) measured in tissues collected from corn hybrid control line 91.

Growth Stage	Tissue	Event	Mean of Cry35Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of Cry35Ab1 (ng/mg Tissue Dry Weight) [1]	Number of Samples[2]
V9	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	5 / 5
R1	Leaf	91	0	0	0 - 0	6 / 6
	Pollen	91	0	0	0 - 0	5 / 5
	Stalk	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	6 / 6
R4	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Forage	91	0	0	0 - 0	6 / 6
Maturity	Grain	91	0	0	0 - 0	6 / 6
R6	Leaf	91	0	0	0 - 0	5 / 5
	Root	91	0	0	0 - 0	5 / 5
	Whole Plant	91	0	0	0 - 0	5 / 5

[1] Sample LOQ for Cry35Ab1: 0.36 ng/mg dry weight for leaf, 0.072 ng/mg dry weight for pollen, 0.18 ng/mg dry weight for stalk, and 0.09 ng/mg dry weight for root and 0.072 ng/mg dry weight for grain and whole plant tissues

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

Mean expression levels of the Cry34Ab1 protein across tissues ranged from 31.5 ng/mg tissue dry weight in V9 whole plant to 220 ng/mg tissue dry weight in R4 leaf. The Cry34Ab1 protein was detected in all tissues assayed for the corn hybrid line 59122 (Table 21).

Table 21. Summary of expression levels of Cry34Ab1 protein (ng/mg tissue dry weight) measured in tissues collected from corn hybrid 59122 (event DAS-59122-7).

Growth Stage	Tissue	Event	Mean of Cry34Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of Cry34Ab1 (ng/mg Tissue Dry Weight) [1]	Number of Samples[2]
V9	Leaf	DAS-59122-7	49.5	7.79	37.0 - 81.4	30 / 0
	Root	DAS-59122-7	38.8	8.28	24.6 - 56.3	18 / 0
	Whole Plant	DAS-59122-7	31.5	15.5	8.67 - 51.9	6 / 0
R1	Leaf	DAS-59122-7	80.6	12.4	59.1 - 103	30 / 0
	Pollen	DAS-59122-7	74.4	6.57	62.9 - 87.2	30 / 0
	Stalk	DAS-59122-7	32.9	4.14	25 - 40.6	30 / 0
	Root	DAS-59122-7	36.8	8.54	23.3 - 52.1	30 / 0
	Whole Plant	DAS-59122-7	45.4	13.5	35 - 71.9	6 / 0
R4	Leaf	DAS-59122-7	220	37.5	143 - 302	18 / 0
	Root	DAS-59122-7	49.1	9.23	33.3 - 67.3	18 / 0
	Forage	DAS-59122-7	53.1	19.1	30.5 - 82.6	6 / 0
Maturity	Grain	DAS-59122-7	49.7	16.2	28.9 - 84.8	30 / 0
R6	Leaf	DAS-59122-7	163	83.6	4.26 - 296	18 / 0
	Root	DAS-59122-7	49.7	19.6	25.7 - 102	18 / 0
	Whole Plant	DAS-59122-7	76.5	10.3	60.5 - 88	6 / 0

[1] Sample LOQ for Cry34Ab1: 0.18 ng/mg dry weight for leaf, 0.36 ng/mg dry weight for pollen, 0.12 ng/mg dry weight for stalk, 0.09 ng/mg dry weight for root, and 0.072 ng/mg dry weight for grain and whole plant tissues.

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

Mean expression levels of the Cry35Ab1 protein across tissues ranged from 0.02 ng/mg tissue dry weight in pollen to 85.3 ng/mg tissue dry weight in R4 leaf. The Cry35Ab1 protein was detected in all tissues assayed for the corn hybrid line 59122 (Table 22).

Table 22. Summary of expression levels of Cry35Ab1 protein (ng/mg tissue dry weight) measured in tissues collected from corn hybrid 59122 (event DAS-59122-7).

Growth Stage	Tissue	Event	Mean of Cry35Ab1 (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of Cry35Ab1 (ng/mg Tissue Dry Weight) [1]	Number of Samples[2]
V9	Leaf	DAS-59122-7	40.7	7.29	29.7 - 55.1	30 / 0
	Root	DAS-59122-7	8.06	2.98	4.08 - 15.4	18 / 0
	Whole Plant	DAS-59122-7	7.36	2.19	4.13 - 10.1	6 / 0
R1	Leaf	DAS-59122-7	52.2	12.9	29.2 - 80.8	30 / 0
	Pollen	DAS-59122-7	0.02	0.04	0 - 0.15	30 / 26
	Stalk	DAS-59122-7	10.0	2.26	5.64 - 14.2	30 / 0
	Root	DAS-59122-7	5.08	1.57	2.49 - 8.85	30 / 0
	Whole Plant	DAS-59122-7	12.3	3.54	9.02 - 18.1	6 / 0
R4	Leaf	DAS-59122-7	85.3	18.9	61.1 - 126	18 / 0
	Root	DAS-59122-7	3.50	0.85	1.74 - 5.76	18 / 0
	Forage	DAS-59122-7	12.4	2.77	8.44 - 16.4	6 / 0
Maturity	Grain	DAS-59122-7	0.99	0.33	0.48 - 1.58	30 / 0
R6	Leaf	DAS-59122-7	54.4	22.2	1.41 - 77.3	18 / 0
	Root	DAS-59122-7	3.10	2.43	0.72 - 10.6	18 / 0
	Whole Plant	DAS-59122-7	13.9	1.91	10.7 - 16.4	6 / 0

[1] Sample LOQ for Cry35Ab1: 0.36 ng/mg dry weight for leaf, 0.072 ng/mg dry weight for pollen, 0.18 ng/mg dry weight for stalk, and 0.09 ng/mg dry weight for root and 0.072 ng/mg dry weight for grain and whole plant tissues

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

V.D.4. PAT protein expression in plant tissues

No PAT protein was detected in the any of the tissue samples of the control line 91 (Table 23).

Table 23. Summary of expression levels of PAT protein (ng/mg tissue dry weight) measured in tissues collected from corn hybrid control line 91.

Growth Stage	Tissue	EVENT	Mean of PAT (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of PAT (ng/mg Tissue Dry Weight) [1]	Number of Samples [2]
V9	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	5 / 5
R1	Leaf	91	0	0	0 - 0	6 / 6
	Pollen	91	0	0	0 - 0	6 / 6
	Stalk	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Whole Plant	91	0	0	0 - 0	6 / 6
R4	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	6 / 6
	Forage	91	0	0	0 - 0	6 / 6
Maturity	Grain	91	0	0	0 - 0	6 / 6
R6	Leaf	91	0	0	0 - 0	6 / 6
	Root	91	0	0	0 - 0	5 / 5
	Whole Plant	91	0	0	0 - 0	6 / 6

[1] Sample LOQ for PAT: 0.15 ng/mg dry weight for leaf, 0.30 ng/mg dry weight for pollen, and 0.06 ng/mg dry weight for stalk, root, grain and whole plant tissues.

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

Mean expression levels of the PAT protein across tissues in hybrid line 59122 ranged from below the LOQ in pollen, forage, grain and R6 whole plant samples to 11.2 ng/mg tissue dry weight in R1 leaf (Table 24).

Table 24. Summary of expression levels of PAT protein (ng/mg tissue dry weight) measured in tissues collected from corn hybrid 59122 (event DAS-59122-7).

Growth Stage	Tissue	EVENT	Mean of PAT (ng/mg Tissue Dry Weight)	Standard Deviation	Min/Max Range of PAT (ng/mg Tissue Dry Weight) [1]	Number of Samples [2]
V9	Leaf	DAS-59122-7	11.1	3.68	5.61 - 19.2	30 / 0
	Root	DAS-59122-7	0.47	0.15	0.27 - 0.87	18 / 0
	Whole Plant	DAS-59122-7	0.18	0.13	0 - 0.40	6 / 1
R1	Leaf	DAS-59122-7	11.2	3.49	6.36 - 18.2	30 / 0
	Pollen	DAS-59122-7	0	0	0 - 0	30 / 30
	Stalk	DAS-59122-7	0.13	0.03	0.07 - 0.20	30 / 0
	Root	DAS-59122-7	0.27	0.12	0.11 - 0.62	30 / 0
	Whole Plant	DAS-59122-7	0.13	0.23	0 - 0.58	6 / 4
R4	Leaf	DAS-59122-7	8.13	3.02	0 - 14.2	18 / 1
	Root	DAS-59122-7	0.09	0.12	0 - 0.34	18 / 9
	Forage	DAS-59122-7	0	0	0 - 0	6 / 6
Maturity	Grain	DAS-59122-7	0	0	0 - 0	30 / 30
R6	Leaf	DAS-59122-7	0.38	0.46	0 - 1.33	18 / 9
	Root	DAS-59122-7	0.08	0.11	0 - 0.46	18 / 9
	Whole Plant	DAS-59122-7	0	0	0 - 0	6 / 6

[1] Sample LOQ for PAT: 0.15 ng/mg dry weight for leaf, 0.30 ng/mg dry weight for pollen, and 0.06 ng/mg dry weight for stalk, root, grain and whole plant tissues.

[2] Number of Samples: number of samples analyzed/ number of samples below sample LLOQ

V.E. Grain and Forage Composition

Grain and forage samples taken from the 2002/2003 field study conducted in Chile were also analyzed for their proximate content (Essner, 2003; Appendix 2, Section 2). The study consisted of six separate field trials located in Chile in which genetically modified hybrid line 59122 and its near isoline EE05F/GR581 (also referred to as 91) were grown. Nutrient composition analyses were conducted at EPL Bio-Analytical Services (Harristown, IL). Nutrient composition data were statistically analyzed to test for differences between the individual modified hybrid (sprayed and non-sprayed) and the control hybrid. Two statistical analyses were performed: The first analysis was a combined analysis of variance (ANOVA) including data combined from each location by analyte that included all 3 replicates of each entry from the 6 separate locations. The combined analysis across all 6 locations used the following mixed model to describe the data (random effects indicated in italics):

Response = site *rep(site)* entry site*entry *residual*

If significant differences were observed in the combined analysis, a single site analysis was conducted to determine if there was a consistent trend where the difference was observed among all locations. The single-site analysis tested for differences between the

individual modified hybrid (sprayed and non-sprayed) and the control hybrid using data from the 3 replicates at each individual location. The single-site analysis used the following mixed model:

Response = rep entry *residual*

The standard error of the means was computed for each entry. Significant differences between the non-sprayed test entry vs. the control were identified using an ANOVA at a 5% level of significance. The results are presented in Tables 25 and 26.

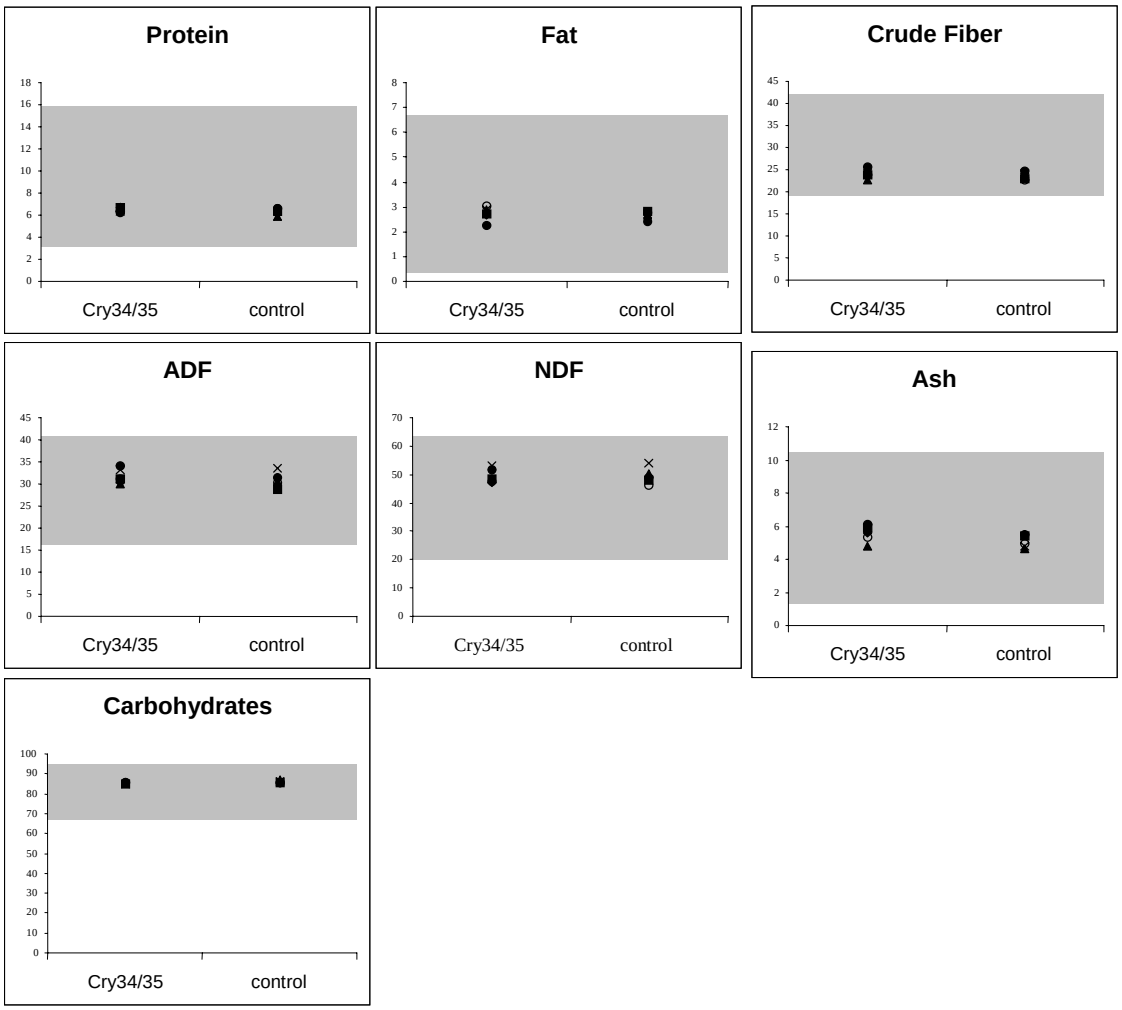
No statistically significant differences were observed for crude fat, ADF, NDF, crude protein and crude fiber in the across location summary analysis (Table 25). Ash and carbohydrate means across locations in the modified hybrid were within 9.2% of the control entry and statistictally different (P<0.05). Significant differences for these analytes were only observed at 3 out of 6 locations for these analytes (data not presented). The across location mean values for all forage proximate, fiber, and carbohydrate analytes for the modified and control entries were within reported literature ranges (Figure 66).

Table 25. Summary of proximates and fiber analysis for DAS-59122-7 and control forage: across sites BU01, BU02, LI01, LI02, VI01, and VI02 in Chile.

Analyte[1]	Literature Range[2]	Means[3]		
		DAS-59122-7	Control	Standard Error
Crude Protein	3.14 – 15.9	6.45	6.27	0.097
Crude Fat	0.37 – 6.7	2.73	2.68	0.068
Crude Fiber	19 - 42	24.0	23.7	0.237
ADF[4]	16.1 – 41.0	31.7	31.1	0.363
NDF[5]	20.3 – 63.7	49.4	49.4	0.388
Ash	1.3 – 10.5	5.60*	5.13	0.103
Carbohydrates[6]	66.9 – 94.5	85.2*	85.9	0.210

[1]Percent of dry weight
[2]Watson, 1982; ILSI, 2003.
[3]Least square means
[4]Acid Detergent Fiber
[5]Neutral Detergent Fiber
[6]Carbohydrates are calculated using the following formula = 100% - % protein - % fat - % ash
*Means in row differ (P<0.05).

Figure 66. Proximates in forage (% dry-weight) in corn line 59122. Means at each location shown: diamond = BU01, square = BU02, triangle = LI01, X = LI02, open circle = VI01, solid circle = VI02. Literature ranges are shaded. ADF=acid detergent fiber. NDF= neutral detergent fiber.



In the proximate analysis of grain, no statistically significant differences were observed for crude fat, crude fiber, ADF, or NDF in the across location summary analysis (Table 26). Crude protein, ash and carbohydrates means across locations in the modified hybrid were within less than 9.2% of the control entry, and significantly different ($P<0.05$). Significant differences for these analytes were observed in 1 of 6 locations for each of the analytes. The across location mean values for all grain proximate, fiber, and carbohydrate analytes for the modified and control entries were within reported literature ranges (Figure 67).

Table 26. Summary of proximates and fiber analysis for DAS-59122-7 and control grain: Across sites BU01, BU02, LI01, LI02, VI01, and VI02 in Chile.

Analyte[1]	Literature Range[2]	Means[3]		
		DAS-59122-7	CONTROL	STANDARD
Crude Protein	6 – 16.1	10.0*	9.61	0.140, 0.136
Crude Fat	1.2 – 18.8	4.69	4.49	0.099, 0.095
Crude Fiber	1.6 – 5.5	2.3	2.3	0.113, 0.109
ADF[5]	1.82 – 11.3	3.5	3.5	0.047, 0.045
NDF[6]	3.0 – 22.6	10.8	10.3	0.301, 0.290
Ash	0.62 – 6.28	1.55*	1.42	0.039, 0.038
Carbohydrates[7]	63.3 – 89.8	83.8*	84.5	0.195, 0.188

[1]Percent dry weight
[2]Watson, 1982 and 1987; Jugenheimer, 1976; OECD, 2002; ILSI, 2003; Essner, 2003.
[3]Least square means
[4]Standard error of DAS-59122-7 is followed by the control – sample missing at location VIO2.
[5]Acid Detergent Fiber
[6]Neutral Detergent Fiber
[7]Carbohydrates are calculated using the following formula = 100% - % protein - % fat - % ash
*Means in row differ (P<0.05).

Figure 67. Proximate analysis of grain (% dry weight) from corn line 59122 and the control line. The data was generated from analysis of samples collected from six field trials conducted in Chile in 2002/2003. Means at each location shown: diamond = BU01, square = BU02, triangle = LI01, X = LI02, open circle = VI01, solid circle = VI02. Literature ranges are shaded. ADF=acid detergent fiber. NDF= neutral detergent fiber.

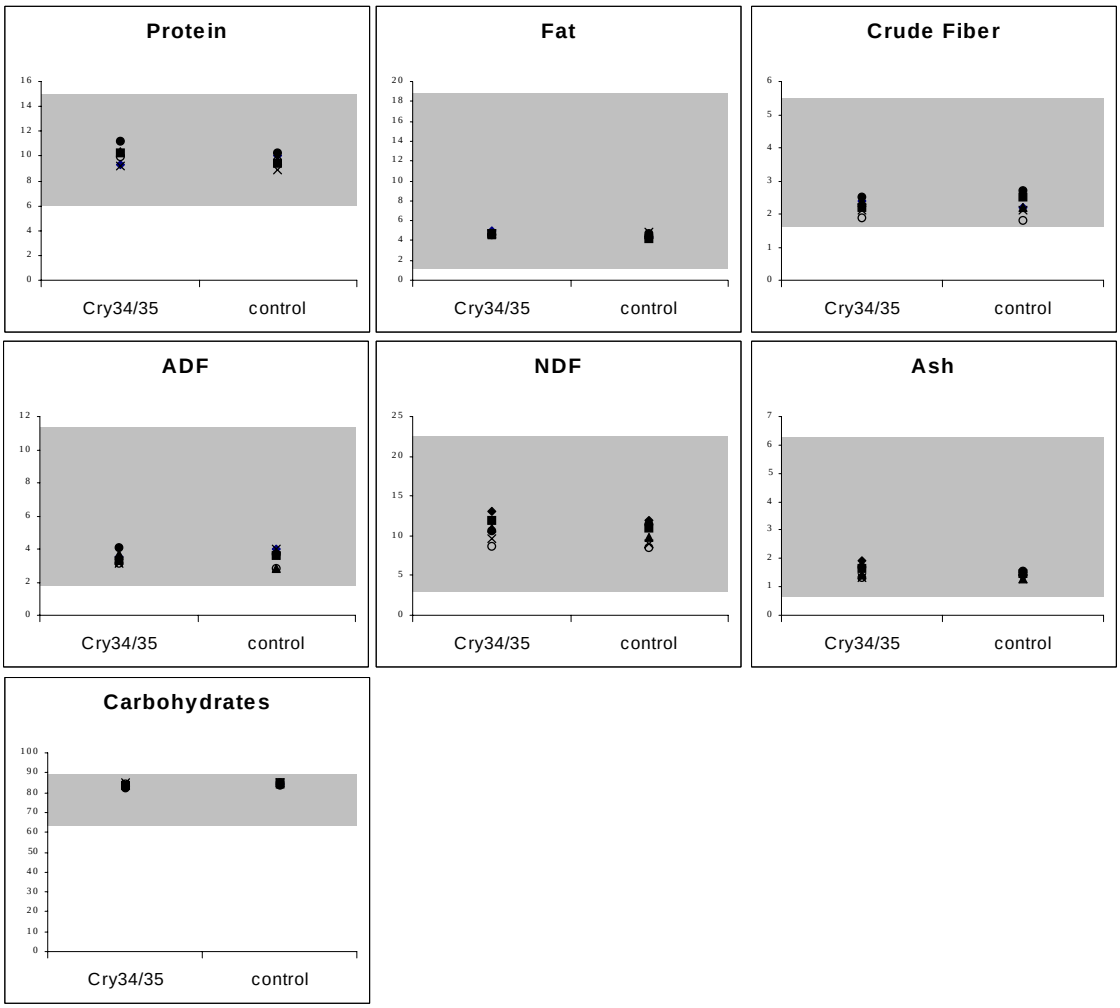


Table 27 summarizes all the data that has been presented to the US Food and Drug Administration (FDA) for the pre-market biotechnology notification (PBN) for line 59122.

Table 27. Summary of compositional analytes measured for grain and forage of line 59122.

Grain			Forage
Proximates	***Vitamins***	***Amino Acids (Essential)***	***Proximates***
Fat	*Vitamin A*	*Methionine*	*Fat*
Protein	*Vitamin B1*	*Cysteine*	*Protein*
Crude Fiber	*Vitamin B2*	*Lysine*	*Crude Fiber*
ADF	*Folic Acid*	*Tryptophan*	*ADF*
NDF	*Vitamin E*	*Threonine*	*NDF*
Ash	*Tocopherols (total)*	*Isoleucine*	*Ash*
Carbohydrates	***Secondary Metabolites***	*Histidine*	*Carbohydrates*
Minerals	*Inositol*	*Valine*	***Minerals***
Calcium	*Raffinose*	*Leucine*	*Calcium*
Phosphorus	*Furfural*	*Arginine*	*Phosphorus*
Copper	*P-Coumaric acid*	*Phenylalanine*	
Iron	*Ferulic acid*	*Glycine*	
Magnesium	***Anti-Nutrients***		
Manganese	*Phytic acid*		
Potassium	*Trypsin Inhibitor*		
Sodium	***Amino Acids (Non-essential)***		
Zinc	*Alanine*		
Fatty Acids	*Aspartic acid*		
Palmitic acid	*Glutamic acid*		
Stearic acid	*Proline*		
Oleic acid	*Serine*		
Linoleic acid	*Tyrosine*		
Linolenic acid			

V.F. Agronomic, Disease and Pest Characteristics

B.t. Cry34/35Ab1 corn line 59122 was evaluated in the field in 2001-2003 in California, Iowa, Illinois, Indiana, Missouri, Wisconsin, Hawaii, Nebraska, Texas and the territory of Puerto Rico under authorizations granted by APHIS (Attachment 1). The purpose of the trials was to evaluate agronomic performance, efficacy against the target pests, increase seed for research purposes, advance generations and/or to evaluate segregation ratios of the event. The field trials were monitored and observed by personnel familiar with corn cultivation practices (breeders, field station managers, field agronomists, field associates).

The personnel conducting the field tests visually monitored plant disease (Northern corn leaf blight, Southern corn leaf blight, Southern rust, grey leaf spot, Stewart's wilt, smut)

and pest resistance (European corn borer, corn earworm, fall armyworm, thrips, aphids, red spider mites) characteristics of *B.t.* Cry34/35Ab1 corn line 59122 and non-modified corn lines. There were no differences reported in severity of disease symptoms or insect damage (other than the targeted organisms susceptible to the Cry34/35Ab1 ICP) between the transgenic plants and the non-modified plants.

V.F.1. Efficacy of event DAS-59122-7 against corn rootworm pests

Biological Spectrum of Activity

The biological activity of Cry34/35Ab1 ICP to a range of insects that feed on corn plants was measured. The microbially-expressed Cry34Ab1 (TSN102172) and Cry35Ab1 (TSN102171) proteins were used in a 1:3 mass ratio (Herman, 2000; Appendix 2, Section 17). The microbially-expressed ICP was shown to be equivalent to the plant-expressed ICP (see Section V.D.). Eight types of insects were chosen for the study based on their pest status on corn and their taxonomic diversity. The insects evaluated were European corn borer (ECB) (*Ostrinia nubialis*), black cutworm (BCW) (*Agrotis ipsilon*), corn earworm (CEW) (*Helicoverpa zea*), southern corn rootworm (sCRW) (*Diabrotica undecimpunctata howardi*), western corn rootworm (wCRW) (*Diabrotica virgifera virgifera*), northern corn rootworm (nCRW) (*Diabrotica barberi*) and corn leaf aphid (CLA) (*Rhopalosiphum maidis*). wCRW was tested as both neonates and adults. The test system was diet overlay for the chewing insects and a membrane feeding system for the CLA.

The results from the bioassays are given in Table 28. There was no statistical difference between the mortality observed for wCRW adults or CLA at the highest concentration tested, and the mortality observed for the negative controls. Less than 25% mortality occurred with the Lepidopterous larvae (ECB, CEW, BCW) at any concentration tested. Only the *Diabrotica* larvae (wCRW, sCRW, nCRW) experienced high mortality. Growth inhibition was a more sensitive measure of toxicity than mortality and was useful for measuring the relative susceptibility of the various insects to the ICP. The relative susceptibilities of the insects to Cry34/35Ab1 ICP were:

(most susceptible) wCRW, nCRW, sCRW>ECB, CEW>>wCRW adult, CLA, BCW (least susceptible)

Table 28. Potency of microbially-expressed Cry34/35Ab1 ICP against several insect pests of corn. Microbially-expressed Cry34Ab1 (TSN102172) and Cry35Ab1 (TSN102171) proteins were used in a 1:3 mass ratio in diet overlay (for chewing insects) bioassays and a membrane feeding system for corn leaf aphid.

Potency of Microbial ICP Against Several Insect Pests of Maize

Insect	GI_{50} (95% Confidence Limits)	LC_{50} (95% Fiducial Limits)
	(µg ai/cm^2)	(µg ai/cm^2)
northern corn rootworm (nCRW) larvae	0.100 (0.0077-1.30)	5.56 (1.76-19.6)
western corn rootworm (wCRW) larvae	2.35 (1.12-4.91)	44.5 (18.5-165) [a]
southern corn rootworm (sCRW) larvae	0.224 (0.089-0.563)	343 (190-796)
European corn borer (ECB) larvae	18.7 (5.58-62.5) [a]	>400
corn earworm (CEW) larvae	42.7 (26.4-69.2)	>400
black cutworm (BCW) larvae	>400	>400
western corn rootworm (wCRW) adult	--	>400
	(µg ai/mL)	(µg ai/mL)
corn leaf aphid (CLA) mixed	--	>4,000

[a] Results from all concentrations were not used in calculations.

Field Efficacy

Efficacy of the Cry34/35Ab1 corn line 59122, along with lines 45216 and 45214, were evaluated for resistance to western corn rootworm (wCRW) at 4 locations in 4 states (IA, NE, WI, IN) in 2003 under USDA APHIS notification #03-035-15n. At each location the efficacy trial was planted into ground that was previously planted with corn to ensure that high numbers of western corn rootworm eggs were present. Additionally, western corn rootworm eggs were infested into the plot at a rate of approximately 1000 eggs per linear foot of row. This artificial infestation of western corn rootworm eggs occurred between the V2 and V4 (2-4 leaf) corn stage. Also at this time, the plots containing Cry34/35Ab1 corn were sprayed with a glufosinate-ammonium herbicide. Cry34/35Ab1 corn is also tolerant to glufosinate-ammonium herbicide, and this tolerance allows for easy identification and subsequent removal of null plants from the plots. Following infestation and removal of null plants, the plots were maintained using corn production methods typical for each location. Just prior to anthesis, the aerial portion of the plants were removed and discarded. The roots were dug from the soil, washed to remove the soil, and then evaluated for western corn rootworm damage using the Iowa State University 0-3 corn rootworm rating scale (J. D. Oleson 1998). The root rating data from each location were pooled across the 4 locations. These trials were designed as randomized complete blocks with three replicates. Data were analyzed using analysis of variance (ANOVA). Data were pooled and analyzed across locations using a mixed factor ANOVA. All means were separated using a protected LSD test (P=0.05).

As expected, corn line 59122 (event DAS-59122-7) as well as corn events A and B expressing the Cry34Ab1/Cry35Ab1 bICP, controlled *Diabrotica* larvae (Table 29). Root ratings for the non-transgenic control plants exposed to western corn rootworm averaged 1.58 (0-3 scale), while the root ratings for the transgenic events ranged from 0.02-0.10 for

western corn rootworm.

Table 29. Efficacy of Cry34/35Ab1-corn rootworm protected corn for control of western corn rootworm pooled across 4 replicated field trial locations. At each location the efficacy trial was planted into ground that was previously planted with corn to ensure that high numbers of western corn rootworm eggs were present. Additionally, western corn rootworm eggs were infested into the plot at a rate of approximately 1000 eggs per linear foot of row at the V2 and V4 corn stage. Just prior to anthesis, the aerial portion of the plants were removed and discarded. The roots were dug from the soil, washed to remove the soil, and then evaluated for western corn rootworm damage using the Iowa State University 0-3 corn rootworm rating scale. The root rating data from each location were pooled across the 4 locations. Data were analyzed using analysis of variance (AOV). Data were pooled and analyzed across locations using a mixed factor AOV. All means were separated using a protected LSD test (P=0.05). Event A and Event B are Cry34/35Ab1 events which are not germaine to this petition. Data were collected from trials conducted under USDA APHIS notification #03-035-15n.

Corn Line	Huxley NE		York NE		Arlington WI		Fowler IN		Average	
Non-Bt Hybrid	1.94	a*	0.93	a	1.03	a	2.43	a	1.58	a
Force	0.15	d	0.11	b	0.19	c	0.57	de	0.25	b
Counter	0.02	d	0.10	b	0.09	c	0.27	e	0.12	b
Event A	0.02	d	0.03	b	0.02	c	0.03	e	0.02	b
Event B	0.07	d	0.03	b	0.03	c	0.10	e	0.05	b
DAS-59122-7	0.03	d	0.04	b	0.05	c	0.09	e	0.05	b

*Means followed by the same letter are not significantly different.

A northern corn rootworm efficacy trial which included the Cry34/35Ab1 corn line 59122 was conducted at Lamberton MN under USDA APHIS notification # 03-052-09n. Plot size in the trial was 3 rows by 20 feet. The trial was organized as a randomized complete block trial with 5 replicates. The test area was located on land naturally infested with northern corn rootworm (nCRW), however, western corn rootworm were also present. The trial was planted on the 23rd of May 2003. Ten one-pint soil samples were collected from the plot area prior to planting and the number of rootworm eggs per pint was determined. While it is possible to discriminate between nCRW and wCRW eggs based on the morphology of the egg chorion, this was not done and so the counts reflect a mixture of both nCRW and wCRW eggs. An average egg density 9.8 egg/pint of soil was measured. Five subsamples of one root each from each plot were rated and plot means were separated using a protected LSD test (p=0.05). Roots were rated for rootworm feeding damage using a modified 1-6 Iowa root rating scale (Table 30).

Table 30. Modified 1-6 corn rootworm damage scale used in the northern corn rootworm efficacy trial at Lamberton MN.

First Generation ECB Leaf Damage Rating	
Rating	**Description**
1	No visible damage.
2	Slight feeding damage on one or more roots. No roots pruned to within 1.5 inches of the stalk.
3	1-5 roots pruned to within 1.5 inches of the stalk
4	One whorl of roots pruned to within 1.5 inches of the stalk
5	Two whorls of roots pruned to within 1.5 inches of the stalk
6	Three whorls of roots pruned to within 1.5 inches of the stalk

As expected, corn lines 59122 (event DAS-59122-7), Event A and Event B, expressing the Cry34Ab1/Cry35Ab1 bICP, controlled nCRW, another *Diabrotica.* Root ratings for the non-transgenic control plants exposed to northern corn rootworm averaged 4.38 (1-6 scale), while the root ratings for the transgenic events ranged from 2.5-2.9 for northern corn rootworm (Table 31).

Table 31. Root ratings for events tested in a northern corn rootworm trial at Lamberton MN. Five subsamples of one root each from each plot were rated and plot means were separated using a protected LSD test (p=0.05). Roots were rated for rootworm feeding damage using a modified 1-6 Iowa root rating scale. Event A and Event B are corn lines containing Cry34/35Ab1 events which are not germaine to this petition. Data was collected from a field trial conducted under USDA APHIS notification 03-052-09n.

Corn Line	Average Root Rating (1-6 scale)	
Non-Bt Hybrid	4.38	a*
Event A	2.90	b
Event B	2.50	b
DAS-59122-7	2.54	b

*Means followed by the same letter are not significantly different.

Efficacy Against Lepidopteran Corn Pests

The efficacy of corn line 59122 was also tested against black cutworm (BCW) and European corn borer (ECB), lepidopteran pests of corn. The efficacy against BCW was evaluated at one location (Indiana) in 2003 under USDA APHIS notification #03-035-15n (Table 32). This trial was planted into 35 cm diameter circular containers on August 26. Approximately 20 kernels were planted into each container. Non-expressing plants were removed from Cry34Ab1/Cry35Ab1 containers (by spraying with glufosinate-ammonium herbicide) on September 4 and the number of remaining plants in each container was recorded. Plants were at the 1 leaf (V1) stage on this date. Third- instar BCW larvae were infested into each container at the rate of 1 larva per plant on September 4. BCW larvae feed on the foliage of developing corn plants and often completely sever the stalk of the plant. This damage is referred to as cutting, and may result in plant death. On September 5, 8, 11 and 18, stand loss due to BCW cutting

damage was measured for each container. The data collected were analyzed using a randomized complete block analysis of variance procedure. Means were separated using Fisher's protected LSD (p=0.05).

Table 32. Efficacy of Cry34Ab1/Cry35Ab1 hybrid for control of black cutworm. The experiment was conducted in circular containers and each container was infested with black cutworm larvae at the V1 growth stage. The data collected were analyzed using a randomized complete block analysis of variance procedure. Means were separated using Fisher's protected LSD (p=0.05). Event A and Event B are corn lines containing Cry34/35Ab1 events not germaine to this petition. Data was collected from trial planted under USDA APHIS notification 03-035-15n.

Corn Line	September 5 (1 day after infestation)		September 8 (4 day after infestation)		September 11 (7 day after infestation)		September 18 (14 day after infestation)	
Non-Bt Hybrid	16.6	a*	57.9	a	81	a	100	a
Event A	17.4	a	74.9	a	81	a	100	a
Event B	20.3	a	63.4	a	86	a	100	a
59122	13.4	a	56.8	a	69	a	100	a

*Means followed by the same letter are not significantly different**.**

There were no statistically significant differences between corn lines 59122, Event A, Event B and a non-Bt corn hybrid evaluated for susceptibility to BCW feeding damage.

An efficacy trial was conducted with Cry34/35Ab1 corn lines 59122, Hybrid A and Hybrid B to characterize the European corn borer (ECB) susceptibility of these events. The trial was planted as a randomized complete block experiment on August 26, 2003 in Indiana under USDA APHIS notification #03-035-15n. Plots in this trial consisted of a single row planted 6 feet in length. Approximately 10-15 plants were contained within each plot. On September 11, at the two-leaf (V2) stage, these plants were infested with 30-50 neonate ECB. Approximately one week later, on September 17, the foliar injury for 10 plants per plot was evaluated using the Guthrie scale (see text below Table 33). The data collected were analyzed using analysis of variance, and means were separated using Fisher's protected LSD (p=0.05).

As expected, the Cry34/35Ab1 corn lines did not control the lepidopteran pest European corn borer (ECB; Table 33). There were no statistically significant differences between corn lines 59122, Event A, Event B and a non-Bt corn hybrid evaluated for susceptibility to ECB feeding damage.

Table 33. Susceptibility of Cry34/35Ab1-corn rootworm protected corn hybrids to European corn borer infestation. At the two-leaf (V2) stage, plants infested with 30-50 neonate ECB. Approximately one week later, the foliar injury for 10 plants per plot was evaluated using the Guthrie scale. The data collected were analyzed using analysis of variance (ANOVA), and means were separated using Fisher's protected LSD (p=0.05). Event A and Event B are corn lines containing Cry34/35Ab1 events which are not germaine to this petition. Data were collected from a trial conducted under USDA APHIS notification #03-035-15n.

Corn Line		
Non-Bt Hybrid	3.6	a*
Event A	3.1	a
Event B	3.6	a
59122	3.0	a

*Means followed by the same letter are not significantly different.

ECB Leaf Damage Rating[a]	
Rating	**Description**
1	No visible damage.
2	Small amount of shot-hole type lesions on a few leaves.
3	Shot-hole injury common on several leaves.
4	Several leaves with shot-hole and elongated lesions.
5	Several leaves with elongated lesions.
6	Several leaves with elongated lesions about 1 inch (2.5 cm) long.
7	Long lesions common on about half (1/2) of the leaves.
8	Long lesions common on about two-thirds (2/3) of the leaves
9	Long lesions common on most leaves

[a]Guthrie, W.D., F.F. Dicke, and C.R. Neiswander. 1960. Leaf and sheath feeding resistance to the European corn borer in eight inbred lines of dent corn. *Ohio Agric. Res. Dev. Cent. Res. Bull.* 860.

V.F.2. Agronomic characteristics

Agronomic trials, encompassing 19 locations in the US Corn Belt, were conducted by DAS and PHI in 2003 on hybrids containing event DAS-59122-7 under notifications from USDA APHIS. The agronomic trait data collected were representative of the type of data used by commercial corn seed companies. The overall range of values for the measured agronomic parameters were all within the range of values obtained for traditional maize hybrids and did not indicate increased weediness.

DAS Agronomic Trials

Two agronomic trials, encompassing 13 and 12 locations, respectively, in the US Corn Belt, were conducted in 2003. The first trial, designated X2793W was a 113-day relative maturity trial and the second trial, designated X2892W, was a 115-day relative maturity trial. Both trials were conducted by Dow AgroSciences and Mycogen Seeds personnel under the appropriate notifications from USDA-APHIS. A list of trial locations is shown in Table 34.

Table 34. Agronomic performance trials conducted in 2003 in the US Corn Belt under USDA APHIS notifications #03-035-15n and #03-052-09n.

Trial Name	Affiliation	Location	Trial Type
X2792W	Mycogen Seeds	DECATUR, IL	Agronomic
X2793W	Mycogen Seeds	PONTIAC, IL	Agronomic
X2793W	Mycogen Seeds	MACOMB, IL	Agronomic
X2793W	Mycogen Seeds	WAYNE CITY, IL	Agronomic
X2793W	Mycogen Seeds	YORK, NE	Agronomic
X2793W	Mycogen Seeds	WYMAN, IA	Agronomic
X2793W	Mycogen Seeds	HUXLEY, IA	Agronomic
X2793W	Mycogen Seeds	HOLDREGE, NE	Agronomic
X2793W	Mycogen Seeds	SEYMOUR, IL	Agronomic
X2793W	Mycogen Seeds	SULLIVAN, IN	Agronomic
X2793W	Mycogen Seeds	COVINGTON, OH	Agronomic
X2793W	Mycogen Seeds	DAVENPORT, IA	Agronomic
X2793W	Mycogen Seeds	DAVID CITY, NE	Agronomic
X2892W	Mycogen Seeds	DECATUR, IL	Agronomic
X2892W	Mycogen Seeds	PONTIAC, IL	Agronomic
X2892W	Mycogen Seeds	MACOMB, IL	Agronomic
X2892W	Mycogen Seeds	WAYNE CITY, IL	Agronomic
X2892W	Mycogen Seeds	YORK, NE	Agronomic
X2892W	Mycogen Seeds	WYMAN, IA	Agronomic
X2892W	Mycogen Seeds	HUXLEY, IA	Agronomic
X2892W	Mycogen Seeds	SEYMOUR, IL	Agronomic
X2892W	Mycogen Seeds	SULLIVAN, IN	Agronomic
X2892W	Mycogen Seeds	COVINGTON, OH	Agronomic
X2892W	Mycogen Seeds	DAVENPORT, IA	Agronomic
X2892W	Mycogen Seeds	DAVID CITY, NE	Agronomic

For each trial, a randomized complete design was utilized with two replications per location and two row plots. Row length was 20 feet and each row was seeded at 37 seeds per row. Rows were later thinned to a maximum of 32 plants per row. Standard regional agronomic practices were utilized and consistently applied across locations in both trials. Entries in trial X2793W included: DAS-59122-7 x Cry1F 1507 stacked BC2S1 hybrid, Cry1F 1507 BC2S1 isogenic hybrid and a Cry1F 1507 elite hybrid. Entries in trial TraitsX2892W included DAS-59122-7 BC2S1 hybrid, a BC2S1 isogenic hybrid and an elite hybrid. All entries within a given trial had the same female parent. The male parent was of a similar genetic background except that it carried event DAS-59122-7. The BC2S1 event DAS-59122-7 bearing inbred was not completely fixed genetically, thus slight genetic variations unrelated to event DAS-59122-7 were expected. The trait bearing inbreds were all homozygous for their respective trait. Both the Cry1F 1507 and non-transgenic isogenic elite hybrids are commercial hybrids in the US. Data collected are representative of the type of data used by commercial corn seed companies to develop elite hybrids.

Data were collected and analyzed for eleven agronomic characteristics (Tables 35 and 36). The parameters of yield (bushels/acre adjusted for moisture) and grain density (weight in pounds of a bushel of corn adjusted to 15.5% moisture) define reproductive capability of the hybrid. Percent moisture at harvest, accumulated growing degree-days to 50% silk (base 50) define the maturity of the hybrid. The agronomic characteristics of percent stalk lodging, root lodging, dropped ears and top integrity determine the harvestability of a hybrid. Stand count measures seed quality and seasonal growing conditions that affect yield. Plant height, ear height and, to some degree, top integrity provide information about the appearance of the hybrids.

Table 35. Trial X2793W: Agronomic characteristics of a 113-day relative maturity DAS-59122-7/TC1507 BC2S1 hybrid as compared to the TC1507 BC2S1 and TC1507 elite isogenic hybrids.
The trial was conducted under USDA APHIS notifications #03-035-15n and #03-052-09n.

TRAIT	DAS-59122-7 /TC1507 BC2S1 Hybrid	BC2S1 Hybrid Maize TC1507 Near Iso	TC1507 Elite Hybrid	Number of locations	Number of reps	LSD[1]
Yield (bushels per acre)	205.9	203.5	205	13	26	6.1
Moisture (%)	23.2	24.2	22.5	13	26	0.6
Accumulated maize growing degree days to reach 50% silking	1354	1367	1342	4	8	18
Grain density[2]	57.2	57.3	58.8	9	18	1
Plant height (inches)	102	99	100	3	6	4
Ear height (inches)	57.2	57.3	58.8	3	6	6
Final stand count (average number of plants per acre in thousands)	30.3	29.4	29.6	13	26	.6
Stalk lodging[3]	.18	.41	.21	13	26	.95
Root lodging[3]	.12	0	0	13	26	.19
Dropped ears per plot	0.0	0.0	0.0	10	20	0
Top integrity[4]	9	9	9	10	20	0

1 Least Significant Difference at the 0.05 level

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

Table 36. Trial X2892W: Agronomic characteristics of a 115-day relative maturity DAS-59122-7 BC2S1 hybrid as compared to the BC2S1 non-transgenic and the elite non-transgenic isogenic hybrids. The trial was conducted under USDA APHIS notifications #03-035-15n and #03-052-09n.

TRAIT	DAS-59122-7 BC2S1 Hybrid	Non-Transgenic BC2S1 Near Iso	Elite Non-Transgenic Near-Iso hybrid	Number of locations	Number of reps	LSD[1]
Yield (bushels per acre)	200.9	208.9	203.8	12	24	7.2
Moisture (%)	25.7	25.2	24.2	12	24	0.75
Accumulated maize growing degree days to reach 50% silking	1340	1375	1327	3	6	17.0
Grain density[2]	56.9	56.7	57.4	8	16	0.9
Plant height (inches)	101	99	102	2	4	6
Ear height (inches)	48	47	49	2	4	6
Final stand count (average number of plants per acre in thousands)	30.1	30.2	30	12	24	1.3
Stalk lodging[3]	1.98	1.26	1.76	12	24	1
Root lodging[3]	0.13	0	0	12	24	.33
Dropped ears per plot	0	0	0	12	24	0
Top integrity[4]	7	7	7	12	24	1

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

In trials X2793W and X2892W, the primary yield comparison was between the BC2S1 DAS-59122-7 and the BC2S1 non-transgenic isogenic hybrid. This comparison factors out a portion of the genetic variability that is an inherent problem when testing early generation hybrids. The DAS-59122-7 x Cry1F 1507 BC2S1 comparison in trial X2793W indicated that there were no significant yield differences. There were also no significant yield differences between the DAS-59122-7 x Cry1F 1507 BC2S1 hybrid and the elite near isogenic Cry1F 1507 hybrid (Table 35). The DAS59122-7 BC2S1 hybrid in trial X2892W was found to be significantly less yielding than the BC2S1 near isogenic

hybrid, however, there was no significant differnce between the DAS-59122 hybrid and the elite near isogenic hybrid. These differences may be due to either the genetic variability still segregating in the BC2S1 row or environmental variation (Table 35). Grain density is another measure of changes in yield. As with yield, the best comparisons are between the BC2S1 hybrids. The BC2S1 DAS-59122-7 hybrid did not show any significant differences with the BC2S1 isogenic hybrid in either trial (Table 35 and 36). Event DAS-59122-7 does not appear to reduce yield since three of the four comparisons between event DAS59122-7 and Non-transgenic hybrids did not indicate significant yield differences.

Maturity is best compared between the BC2S1 isogenic hybrids in each of these trials. In trial X2793W event DAS-59122-7 BC2S1 hybrid was significantly drier than the non-transgenic hybrid (Table 35), but the same comparison in trial X2892W was not significantly different (Table 36). The event DAS-59122-7 hybrids were also significantly later in maturing than the elite non-trangenic isogenic hybrids. These trends are most likely associated with slight genetic differences in dry down and was expected that hybrids made from inbreds with more backcrosses would not show these differences. Maturity was calculated by gathering data on growing degree units to 50% silk. There were no significant changes observed in flowering between the DAS-59122-7 x Cry1F 1507 BC2S1 hybrid in trial x2793W and its isogenic comparisons (Table 35). The BC2S1 comparison in trial X2892W did indicate that the DAS-59122-7 hybrid was significantly earlier. There was no significant difference between the DAS-59122-7 BC2S1 hybrid and the elite isogenic hybrid. The actual differences in flowering observed were very small and would not affect use patterns in commercial hybrids.
There were no significant differences observed in final stand between any of the comparisons in trial X2892W (Table 36). There were significantly fewer non-transgenic plants than transgenic plants in the X2793W trial (Table 35). The populations in both trials contained more than 29,000 plants per acre indicating that most all of the seeds germinated at all locations. Population changes of the magnitude observed should not have an effect on the quality of the trial.

Plant and ear height were used as indicators of hybrid stature. No significant differences were observed between the three hybrids in either trial, indicating they had a similar appearance. Percent stalk lodging, percent root lodging, dropped ears, and top integrity are all measurements of a hybrid's ability to be harvested. No significant difference was found in either trial for any of these traits (Table 35 and 36).

In conclusion, none of the statistically significant differences observed in comparisons between event DAS-59122-7 hybrids and the isogenic control hybrids were attributable to the transgenic modification of corn. Furthermore none of the differences were biologically or commercially significant. The overall range of values for the measured parameters were all within the range of values obtained for traditional maize hybrids and would not lead to a conclusion of increased weediness.

PHI Agronomic Trial

The trial was planted across the Midwest US corn-belt at 7 locations, with 3 replications planted per location, where seed quantities and space permitted. The trial was conducted by PHI personnel. Table 37 shows the list of trial locations. A random complete block (RCB) design was used; entries were nested by hybrid base genetics and by presence/absence of Cry1F event TC1507. Plots comprised 2 rows each. Row length was 17' and each row was sown with 38 kernels per row. After emergence, rows were thinned to a maximum of 30 plants per row. Standard regional agronomic practices were utilized and consistently applied across all locations. This included an application of ForceTM to control CRW was used at all locations, except at Macomb IL where Lorsban was used.

Table 37. Agronomic performance trial conducted in 2003 at US Corn-Belt locations by Pioneer under USDA APHIS notification #03-022-01R.

Trial Name	Affiliation	Location	Trial Type
RI608D3F	Pioneer	CHAMPAIGN, IL	Agronomic
RI608D3F	Pioneer	PRINCETON, IL	Agronomic
RI608D3F	Pioneer	MACOMB, IL	Agronomic
RI608D3F	Pioneer	MARION, IA	Agronomic
RI608D3F	Pioneer	YORK, NE	Agronomic
RI608D3F	Pioneer	JOHNSTON, IA	Agronomic
RI608D3F	Pioneer	WINDFALL, IN	Agronomic

The quantitative agronomic traits data were collected and analyzed for 8 agronomic characteristics including: yield, moisture, accumulated growing degree-days to 50% silk, accumulated growing degree-days to 50% pollen shed, grain density, plant height, ear height, and final plot density. Least squared means estimates were calculated using SAS and are summarized for these traits in Tables 39 and 40. Three other traits: stalk lodging, root lodging, and staygreen, were also monitored and qualitative notes were recorded for each location.

Within the trial, the primary yield comparisons were between four pairs of BC2S1 hybrid entries. The 05F/21T base genetic background comprised two key comparisons: (1) event DAS-59122-7 vs. a near isogenic null hybrid, both hybrids without event TC1507, and (2) event DAS-59122-7 vs. a near isogenic null hybrid, both hybrids with event TC1507. The other 581/1W2 base genetic background comprises a similar set of two more key comparisons: a pair without Cry1F event TC1507, and a pair of entries with event TC1507. Focus on these key comparisons helps to factor out some of the genetic variability inherent to any test of early generation hybrids.

In general, event DAS-59122-7 containing hybrids produced similar amounts of grain to

their near-isogenic counterparts. Comparisons between least squares means for entries with event DAS-59122-7 and without event DAS-59122-7 showed parity performance in yield, and in some instances yield advantages in hybrids containing event DAS-59122-7 compared to lines without event DAS-59122-7, even after chemical insecticide has been used to control CRW pressure (Table 38). Genetic variability among near-isogenic lines may explain some of the numeric differences observed for yield. In addition, the yield advantage in the 581/1W2 hybrid background may also be explained by the hypothesis that the CRW protection of event DAS-59122-7 goes beyond the protection offered by chemical insecticides – at least under some conditions. A differential in susceptibility to CRW damage (due to physiological and/or morphological differences between the base genetics of 581/1W2 and 05F/21T) might explain why event DAS-59122-7 entries yielded at parity with the entries without event DAS-59122-7 05F/21T entries, whereas among the 581/1W2 entries, event DAS-59122-7 conferred a clear yield advantage. A review of entry means for each location (Table 39) suggests that under some conditions (such as at Princeton - and especially at Macomb, IL) hybrids containing event DAS-59122-7 offered an exciting advantage against CRW pressure that chemical insecticides had failed to control. CRW damage was measured using the 0-3 CRW node injury scale. At each location, scores were assessed midseason from roots in border rows adjacent (and on all four sides) to yield trial; the scores should be viewed as only a crude indicator of the actually CRW pressure within the plots of the yield trial.

Grain density is another measure closely related to yield. As with yield, the best comparisons are between the near-isogenic pairs of BC2S1 hybrids. For grain density, the BC2S1 DAS-59122-7 hybrid line did not show any significant differences with the BC2S1 isogenic hybrid or with the elite hybrid (Table 38).

Similarly, maturity indications, such as moisture, heat units to tasseling, and heat units to silking, showed no obvious relation to the presence or absence of event DAS-59122-7 (Table 38).

Morphologically, presence vs. absence of event DAS-59122-7 affected neither plant height nor ear height. In contrast, plant height was affected by differences between base genetics.

Stalk lodging, percent root lodging, and staygreen are all indicators of a hybrid's ability to be harvested, as well as health indicators. The occurrence of root lodging at one location (Macomb IL) was the most significant contrast to be observed between lines with and without event DAS-59122-7 for the set of qualitative traits that were monitored. At the Macomb IL location, all of the entries without event DAS-59122-7, including the elite hybrid entry, suffered dramatic root lodging- likely due to heavy CRW pressure that was not sufficiently controlled by chemical insecticide. In contrast, no root lodging was observed among the entries with event DAS-59122-7 at the Macomb IL location. At the other 6 trial locations, significant variation between entries with event DAS-59122-7 and without it was not detected for staygreen or lodging. Finally, no significant differences were observed in final stand (Table 38); this uniformity of stand facilitated robust agronomic evaluation of the Cry34/35Ab1 CRW-protected technology.

In conclusion, for most agronomic traits, in the absence of CRW pressure, hybrids containing event DAS-59122-7 perform similarly to conventional maize hybrid performance. Under CRW pressure, however, Cry34/35Ab1 CRW-protected corn (event DAS 59122-7) offers an advantage in yield and root lodging. Furthermore, none of the differences are of biological or agronomical disadvantage. The overall range of values for the measured parameters are all within the range of values obtained for traditional maize hybrids and do not lead to a conclusion of increased weediness.

Table 38. Summary of agronomic performance of BC2S1 hybrids with and without event DAS-59122-7 in PHI agronomic trial. Least square means are presented. The trial was conducted under USDA APHIS notification #03-022-01R.

	BC2S1 hybrid 05F/21T				BC2S1 hybrid 581/1W2				Elite hybrid	No. of locations	No. of reps	LSD[3]
	Without event TC1507		With event TC1507		Without event TC1507		I. With event TC1507					
	59122[1]	null[2]	59122[1]	null[2]	59122[1]	null[2]	59122[1]	null[2]				
Trait	A	B	C	D	E	F	G	H	I			
Yield (bushels per acre)	168.3	171.7	166.3	165.3	159.4	144.0	167.6	153.7	175.9	7	19	12.2
Moisture (%)	24.1	23.9	25.2	25.2	20.0	20.1	21.5	21.8	22.8	7	19	1
Accumulated maize growing degree days to reach 50% pollen shed	1388	1404	1416	1422	1333	1329	1331	1368	1317	6	16	48
Accumulated maize growing degree days to reach 50% silking	1393	1401	1425	1433	1323	1319	1343	1352	1317	6	16	44
Grain density[4]	54.2	55.1	54.5	55.4	55.3	55.7	56.0	55.4	56.2	7	19	1.7
Plant height (inches)	121.9	123.2	124.6	124.5	115.4	115.7	119.7	117.2	114.8	6	16	5.1
Ear height (inches)	51.1	51.8	51.3	53.5	50.4	48.6	52.6	51.3	47.3	6	16	3.6
Final stand count (average number of plants per acre in thousands)	32.6	32.8	32.6	32.5	32.4	32.3	32.8	32.1	32.6	7	19	1.8

1 Hybrid contains event DAS-59122-7.
2 Hybrid does not contain event DAS-59122-7.
3 Least Significant Difference at the 0.05 level
4. Weight (in pounds) of a bushel of grain at 15.5% moisture

Table 39. Yield details and CRW injury ratings for individual locations. Simple means are presented for the replications of each entry from each location tested. The trial was conducted under USDA APHIS notification #03-022-01R.

		BC2S1 hybrid 05F/21T				BC2S1 hybrid 581/1W2				Elite hybrid
		Without event TC1507		With event TC1507		Without event TC1507		II.	With event TC1507	
		59122[1]	null[2]	59122[1]	null[2]	59122[1]	null[2]	59122[1]	null[2]	
Location	CRW pressure after insecticide	A	B	C	D	E	F	G	H	I
MACOMB, IL	0.7	191.1	163.0	179.4	135.3	155.3	127.6	174.2	121.3	156.5
PRINCETON, IL	0.4	179.4	171.9	175.3	177.1	167.7	128.3	157.1	135.3	167.9
WINDFALL, IN	<0.10	133.5	148.1	133.9	146.8	142.4	138.0	149.8	149.4	147.2
MARION, IA	<0.10	152.0	143.1	138.6	149.6	139.0	127.9	132.4	131.8	153.3
YORK, NE	0.3	175.3	177.7	163.3	160.7	176.1	170.1	190.9	185.5	183.2
JOHNSTON, IA	<0.10	183.7	210.3	200.8	203.2	189.2	176.2	203.9	178.4	218.5
CHAMPAIGN, IL	<0.10	171.2	180.9	173.6	175.0	148.8	139.8	165.8	166.7	190.7

1 Hybrid contains event DAS-59122-7.

2 Hybrid does not contain event DAS-59122-7.

V.G. Secondary Metabolites, Anti-Nutrients and the Allergenic Potential of Proteins in Line 59122

Secondary metabolites are neither nutrients nor anti-nutrients (OECD, 2002). Characteristic secondary metabolites in corn are furfural and phenolic acids (ferulic and p-coumaric). Furfural is a heterocyclic aldehyde and is generally recognized as safe (GRAS). The phenolic acids are structural components of plant cells and act as a natural pesticide against insects and fungi. Ferulic acid and p-coumaric acid have weak anti-oxidative properties. Anti-nutrients in corn include phytic acid, raffinose, trypsin and chymotrypsin inhibitors. Raffinose is a non-digestible oligosaccharide and is considered an anti-nutrient due to the gas production and resulting flatulence caused by its consumption. The levels of trypsin and chymotrypsin inhibitors in corn are considered insignificant. Phytic acid binds about 60-75% of phosphorus in the form of phytate

which results in less than 15% bioavailability of phosphorus for nonruminant animals It is common practice for swine and poultry producers to add phytase to the diet formulation to improve the utilization of phosphorus for these nonruminants.

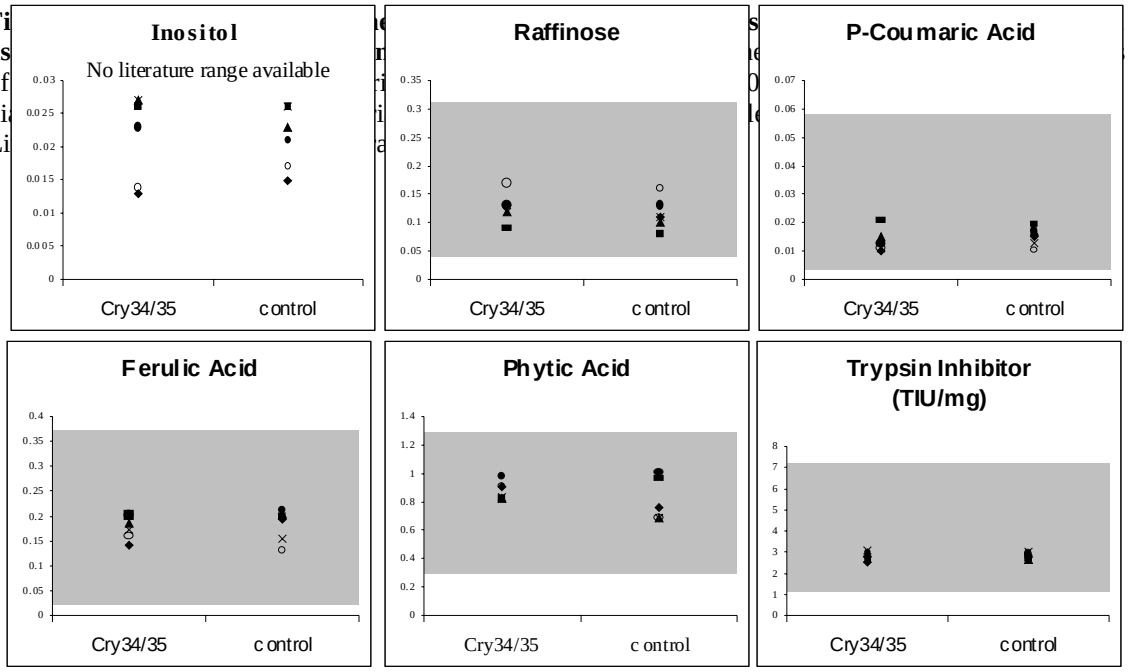
Grain of line 59122 was analyzed for secondary metabolites and anti-nutrients common to corn. Grain samples were collected from the field trial conducted in Chile in 2002/2003 as stated in Section V.E. of this petition. The results of these analyses are presented in Table 40 and Figure 68 and show that line 59122 is comparable to the nontransgenic control.

Table 40. Summary of secondary metabolites and anti-nutrients for DAS-59122-7 and control grain: Across sites BU01, BU02, LI01, LI02, VI01, and VI02 in Chile.

Analyte[1]	Literature Range[2]	Means[3]		
		DAS-59122-7	Control	Standard Error[4]
Secondary Metabolites and Anti-Nutrients				
Inositol	NR[5]	0.022	0.021	0.001
Raffinose	0.08 – 0.31	0.13	0.12	0.007
Furfural	NR[5]	ND	ND	ND
P-Coumaric Acid	0.003 – 0.058	0.014	0.015	0.001
Ferulic Acid	0.02 – 0.37	0.177	0.182	0.012
Phytic acid	0.29 – 1.29	0.877	0.798	0.040, 0.039
Trypsin Inhibitor (TIU/g)	1.1 – 7.18[5]	2.82	2.84	0.065, 0.063

[1]Percent dry weight
[2]Watson, 1982; OECD, 2002; ILSI, 2003.
[3]Least square means
[4]Standard error of DAS-59122-7 is followed by the control – sample missing at location VIO2.
*Means in row differ (P<0.05).

Fi **as** of di Li



Studies were also conducted to ascertain any allergenic potential of the Cry34Ab1, Cry35Ab1 and PAT proteins. These studies included: 1) amino acid sequence homology to known allergens; 2) acute oral toxicity tests; 3) digestive fate in simulated gastric fluid (SGF), and 4) heat lability of the proteins.

For the Cry34Ab1 and Cry35Ab1 proteins, no homology to the sequence of known protein allergens was shown when using a sequence evaluation scheme based on that formulated by Gendal (1998), by the Joint FAO/WHO Expert Consultation (2001), and by the Codex Alimentarius (Codex *Ad HOC* Open-ended Working Group on Allergenicity) (Song, 2003). An immunologically significant sequence identity requires a match of at least eight contiguous identical amino acids or 35% identity over eighty amino acid residues and no matches of this type were found in the ICP.

In *in vitro* studies, Cry34/35Ab1 ICP exhibited a high rate of digestibility (DT_{90} = 6.5 minutes (average) and DT_{97} = <5 minutes for Cry34Ab1 and Cry35Ab1, respectively) under simulated gastric conditions (referred to as SGF) in the presence of pepsin (pH 1.2) (Herman *et al*, 2003). Previous simulated gastric fluid digestion studies for other registered *B.t.* products and for Cry34Ab1 and Cry35Ab1 (Korjagin and Ernest, 2000; Appendix 2, Section 19) focused on the time required for the protein of interest to become undetectable via a specific analytical method (western blotting or SDS-PAGE). This criterion is both a function of protein digestion and the sensitivity of the analytical technique (i.e., western blotting). When the sensitivity of the analytical method (western blotting) is known, it is possible to conclude when the ICP is degraded below the

established assay sensitivity. The limit of detection for Cry35Ab1 by SDS-PAGE analysis was found to be <15.6 ng/lane (Figure 69). Since approximately 0.61 µg/lane of Cry35Ab1 was loaded on the SDS-PAGE gel (based on undigested quantity), the analytical sensitivity of the assay was <2.6% of the amount present prior to digestion. Therefore, Cry35Ab1 was no longer visible on the SDS-PAGE gel after 5 minutes, when greater than 97% of the protein had been digested. Because the data were consistent with rapid digestion and with the profile of other registered plant-incorporated-protectants, more quantitative studies were not undertaken with Cry35Ab1.

A more quantitative approach to measuring digestion rate is the quantification of the residue after exposure to SGF for various time intervals and then modeling the decay (Herman *et al.*, 2003; Brussock and Currier, 1990). Classical enzyme theory (Michaelis-Menten rate law) states that when the enzyme is present at a substantially higher concentration than the substrate, as has been the case for previously reported ICP digestibility studies, the degradation is predicted to be governed by first-order kinetics and thus, point estimates of decay such as half-life or DT_{90} can be calculated (Rawn, 1989). Cry34Ab1 degradation followed classical first-order kinetics and was highly reproducible in two experiments (Figures 70-71). Estimated half-lives were 1.9 and 2.0 minutes and DT_{90} values were estimated at 6.3 and 6.8 minutes. DT_{90} values were calculated since this was the limit of detection in digestion experiments reported with some *B.t.* proteins expressed in currently registered PIPs (Monsanto, 2001; USEPA, 2001) and provided a means for comparing the digestibility of the Cry34Ab1 protein to these proteins. The *B.t.* proteins expressed in all currently registered PIPs become undetectable at or below 7 minutes of SGF exposure. Thus, the demonstrated digestibility of Cry34Ab1 protein is consistent with the rapid digestion reported for the other registered PIPs (DT_{90}<7 minutes) (USEPA, 2001).

Figure 69. SDS-PAGE and western blot of Cry35Ab1 SGF digestion. M = molecular mass markers in kilodaltons. The SDS-PAGE gel was loaded at 610 ng AI/lane (predigestion) and the western blot was loaded at 15 ng AI/lane (predigestion).

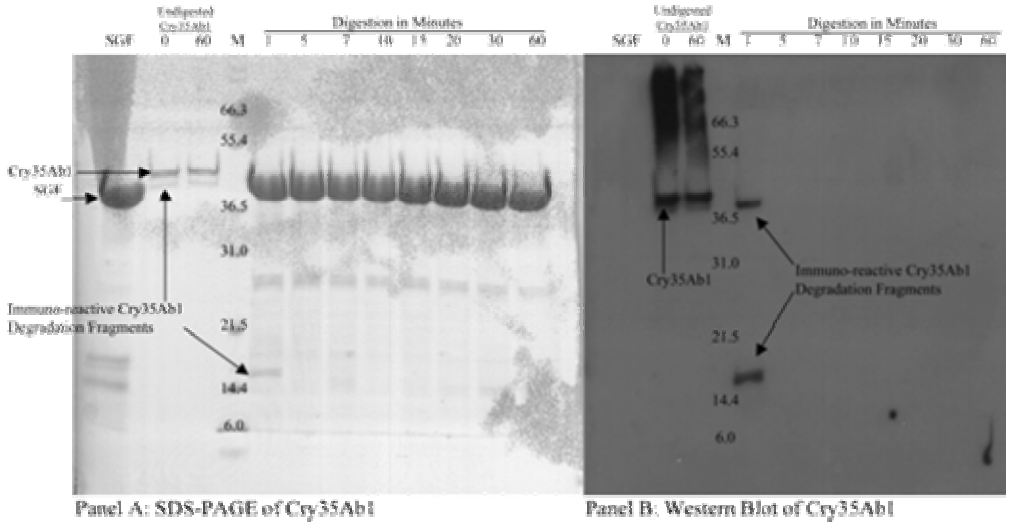


Figure 70. SDS-PAGE gels from Cry34Ab1 digestions. The gel was loaded at 190 ng AI/lane (predigestion). Each row represents a separate experiment (three replicate gels). Note the light background bands at approximately 14 kDa in lanes containing SGF only that are of similar intensity to those seen at later Cry34Ab1 digestion times.

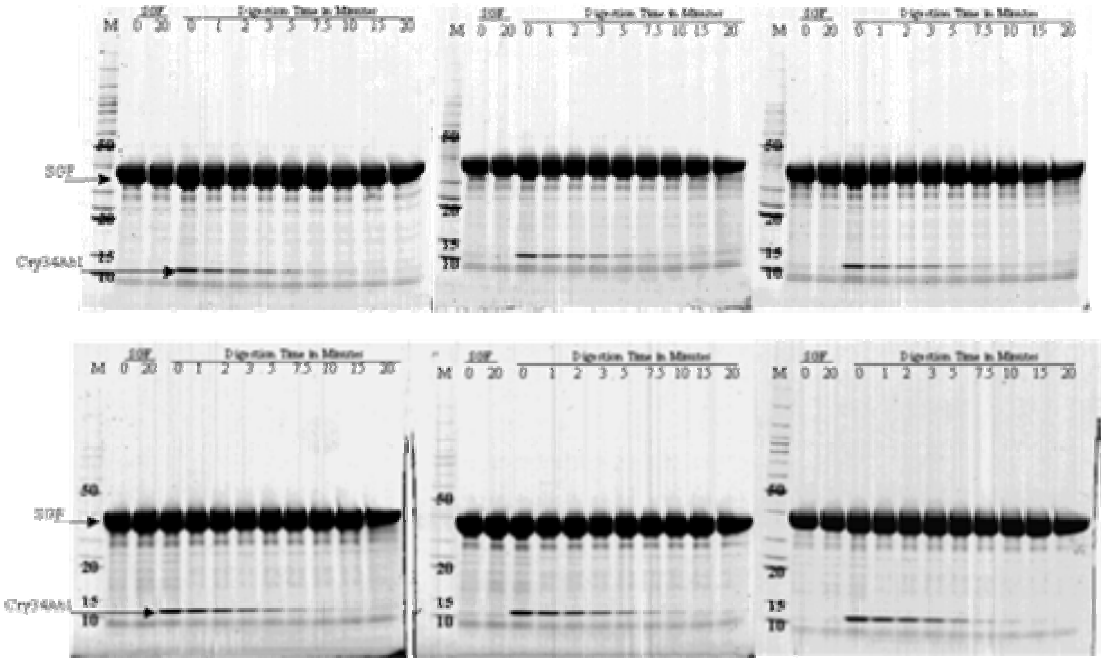
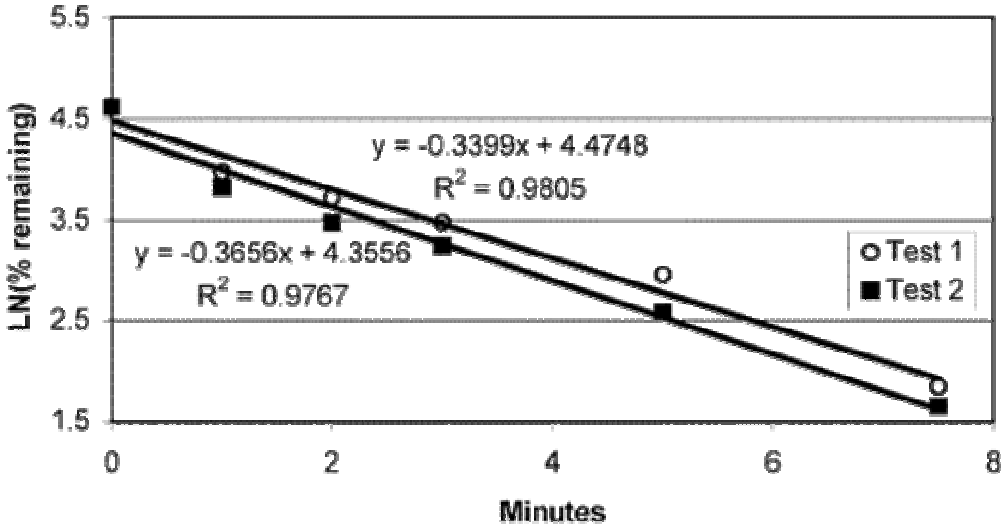


Figure 71. Digestion graph of Cry34Ab1 protein. A first-order decay model was determined by regressing the natural logarithm of the percent remaining (based on SDS-PAGE densitometry estimates) against time.



Heat Lability

Two studies on heat lability of the Cry34/35Ab1 ICP were conducted. In the first study (Herman, 2000; Appendix 2, Section 26) aqueous formulations of the ICP were incubated at 60, 75 or 90°C for 30 minutes. After incubation, neonate southern corn rootworm larvae (*Diabrotica undecimpunctata howardii*; sCRW) were exposed to artificial dietary substrates that had been treated with the ICP incubated samples. Insect mortality and weight were measured after 6 days and growth inhibition was calculated based on comparison with negative controls. The results of this experiment indicated that the ICP was deactivated after exposure to the temperatures. A second study (Herman, 2002; Appendix 2, Section 28) was conducted to examine the heat lability of the individual component proteins (Cry34Ab1 and Cry35Ab1) of the ICP by fortifying heated samples of the ICP with non-heated samples of each component protein. This allowed the heat lability of the complimentary protein to be measured since both proteins are required for maximum activity against corn rootworms (Herman *et al.*, 2002). Based on the significant loss of activity seen with the ICP with and without the non-heated Cry35Ab1 at temperatures of 60, 75 and 90°C, Cry35Ab1 and the ICP demonstrated very significant heat lability. The Cry34Ab1 protein showed similar heat lability in this study, however, some native Cry34Ab1 residue was indicated at 60 and 75°C. This activity was indistinguishable from background effects after exposure to 90°C, indicating substantial heat lability for the Cry34Ab1 protein as well.

Acute Toxicity

Acute toxicity tests were conducted on mice to determine any toxicity of the Cry34/35Ab1 ICP. In the study with Cry34Ab1 protein alone study, a microbial protein

preparation containing 54% Cry34Ab1 protein was evaluated for acute oral toxicity from gavage administration to five male CD1 mice (5000 mg microbial protein preparation (54%)/kg body weight) (Brooks and De Wildt, 2000; Appendix 2, Section 21). All mice survived and there were no adverse effects in terms of body weights, detailed clinical observations, and gross pathological lesions during the two-week observation period. Under the conditions of this study, the LD_{50} of the Cry34Ab1 microbial protein in male and female CD-1 mice was greater than 2700 mg a.i./kg.

In the study with Cry35Ab1 protein alone study, a microbial preparation containing 37% Cry35Ab1 protein was evaluated for acute oral toxicity from gavage administration to five male CD-1 mice (5000 mg microbial protein preparation (37%)/kg body weight) (Brooks and DeWildt, 2000; Appendix 2, Section 22). All mice survived and there were no treatment-related gross pathological observations. Two mice lost weight initially after the gavage administration but gained weight over the remainder of the study and one mouse had fluctuating body weight for the duration of the study. The body weight fluctuation and loss was probably due to gavage with maximum volume of methylcellulose. Under the conditions of this study, the LD_{50} for the pure protein is greater than 1850 mg/kg.

For Cry34Ab1 + Cry35Ab1 study (Brooks and DeWildt, 2000; Appendix 2, Section 23), a mixture (1: 4.6 ratio) of the microbial preparation containing 54% Cry34Ab1 protein and the microbial preparation containing 37% Cry35Ab1 was evaluated for acute oral toxicity from gavage administration to five male and five female CD-1 mice. The mixture was designed to provide a dose of 5000 mg test material/kg body weight that contained 482 mg/kg pure Cry34Ab1 protein and 1520 mg/kg pure Cry35Ab1 protein (a 1:3 ratio to provide an equimolar mixture of the pure proteins). All mice survived and there were no adverse effects in terms of body weights, detailed clinical observations, and gross pathological lesions during the two-week observation period. Under the conditions of this study, the LD_{50} of the Cry34/35Ab1 microbial protein in male and female CD-1 mice was greater than 2000 mg a.i./kg.

The PAT protein is an acetyltransferase and has been well studied and characterized. Acetyltransferases are ubiquitous in nature and are found in microbes, plants and animals. PAT rapidly degrades in elevated temperature. In 1997 EPA issued a final rule exempting PAT from the requirement of a tolerance in all raw agricultural commodities when used as a plant-incorporated-protectant (PIP) inert. (FR April 11, 1997, vol. 62, No. 70). In exempting PAT, EPA evaluated data submitted regarding its behavior in simulated digestive fluid and the acute oral toxicity of the protein. EPA concluded that there is “a reasonable certainty of no harm” that would result from aggregate exposure due to the fact that no toxicity in mammals has been observed from PIPs and PAT itself showed no toxicity (no mortality in the high-dose 2500 mg/kg subgroup of test animals); and that in vitro digestibility data indicated that PAT is rapidly degraded in digestive fluids.

The PAT protein present in transgenic line 59122 has been shown to be biochemically equivalent to the *E. coli*-produced protein which has been used in toxicology and biochemical tests to show its lack of allergenic potential (Schafer *et al.*, 2003; Appendix

2, Section 4). The microbially-derived PAT (84% pure microbial protein) was evaluated for acute oral toxicity by subjecting mice (5 males and 5 females) to 6000 mg/kg body weight of the test material (5000 mg/kg PAT) via oral gavage (Brooks, 2000; Appendix 2, Section 23). All mice survived the two week test period and no treatment-related clinical observations were observed. All mice gained weight during the test period and there were no gross pathological changes. The acute oral LD_{50} was ascertained to be >5000 mg/kg PAT which is consistent with previous studies (EPA, 1997). In simulated gastric fluid (pepsin) digestion, the PAT protein was found to be digested within 5 seconds (Glatt, 1999). No amino acid homology was found between PAT and any known protein allergen (Meyer, 1999).

VI. ENVIRONMENTAL CONSEQUENCES OF INTRODUCTION

VI.A. ESTIMATED ENVIRONMENTAL CONCENTRATION

Expression data indicate that mean protein levels in all plant tissues during the growing season range from 31 to 226 µg/g dw Cry34Ab1 and 0.02 to 83 µg/g dw Cry35Ab1in Event DAS-59122-7 (Tables 19-20, Essner, 2003; Appendix 2, Section 2). Both proteins contribute to activity in target insects, but the concentration response for Cry34Ab1 is much steeper than for Cry35Ab1 (Herman *et al*., 2002). High-end exposure estimates (HEEE) calculated as the upper 90% confidence bound on the mean of Cry34Ab1 protein expression are considered the most appropriate data to relate to ecotoxicology studies. These values range from 32 to 235 µg/g dw for all plant tissues throughout the growing season (Tables 19-20).

VI.B. EXPOSURE TO NON-TARGET ORGANISMS AND HUMANS

Exposure to non-target organisms

Mammals

The results of the acute toxicology studies were discussed in Section V.G. of this petition. USEPA granted a temporary exemption from the requirement of a tolerance in June, 2003 which extends through April 30, 2006 (FR July 7, 2003: Vol. 68, No. 129, p. 40178-40183).

Birds

In a 42-d nutritional equivalency study conducted using commercial broiler chickens, birds fed maize grain expressing Cry34/35Ab1 proteins showed no effects in mortality, weight gain, feed efficiency or carcass yields relative to a non-transgenic, near-isoline of maize or commercial maize (Smith and Hinds, 2003). Expression data for an earlier higher expressing event (event 15344) in starter and grower diets ranged from 13 to 23 ng/mg for Cry34Ab1 and 1.7 to 2.1 ng/mg for Cry35Ab1.

Soil Invertebrates

Earthworm. Microbially-derived Cry34/35Ab1 protein mixture (25.4 mg a.i./kg) showed no toxicity to earthworms (*Eisenia fetida*). The LC_{50} was >6.35 mg Cry34Ab1 a.i./kg dry soil, the highest concentration used (Bryan, *et al.*, 2000; Appendix 2, Section 27). The target multiple of 20X field exposure for dosing was based on the assumption of a Cry34Ab1 expression level of 11.5 µg/g in senescent plant tissue. For Event DAS-59122-7, the HEEE senescent plant value is 109.03 µg/g (Table 41), resulting in an actual multiple of 11.5/109.03 x 20 = 2.1X.

Collembola. Collembola plays a major role in soil ecosystems due to its feeding on decaying plant materials. Therefore, a laboratory study to determine the chronic effects of the Cry34/35Ab1 ICP on survival and reproduction of the soil dwelling invertebrate collembola (*Folsomia candida*) was conducted using microbially-derived Cry34Ab1 (54% a.i.) and Cry35Ab1 (37% a.i.) added to Brewer's yeast (standard food for collembola) to attain a target 10X amount (12.7 mg/kg diet) of the expression of the ICP *in planta* (based on an earlier higher expressing event than the subject event of this petition) (Teixeira, 2001; Appendix 2, Section 34). There was no effect shown from the ICP exposure in the diet. The EC_{50} was >3.18 mg Cry34Ab1 ICP per kg diet. The 10X target multiple assumed a Cry34Ab1 expression level of 11.5 µg/g in senescent plant tissue. For Event DAS-59122-7, the HEEE senescent plant value is 109.03 µg/g (Table 41), resulting in an actual multiple of 11.5/109.03 x 10 = 1.1X.

Aquatic Organisms

Daphnia. There are no known adverse effects of Cry proteins on the aquatic invertebrate *Dapnia magna* Strauss. This was confirmed in a 48-hour static limit test with *Daphnia* was conducted with 100mg/L Cry34/35Ab1 ICP (57 mg/L Cry 34Ab1 and 43 mg/L Cry35Ab1) (Marino and Yaroch, 2001; Appendix 2, Section 32). No adverse effects to the *Daphnia* were observed. The EC_{50} value is greater than 100mg Cry34/35Ab1/L. The predicted Cry34Ab1environmental concentration in a standard EPA farm pond using the senescent plant HEEE expression value for Event DAS-59122-7 (109.03 µg/g) (Table 42) is 0.186 mg/L (Poletika, 2003; Appendix 2, Section 11). While no target multiple was assumed in the daphnid test, the actual multiple achieved can be estimated as 57/0.186 = 306X the high-end expected environmental concentration.

Fish. The acute dietary toxicity of the Cry34/35Ab1 ICP to the rainbow trout (*Onchorynchus mykiss* Waldbaum) was determined for fish exposed for eight days to standard fish diet containing 100 mg a.i./kg diet of the microbially-derived Cry34Ab1 and Cry35Ab1 proteins (25 mg/kg Cry34Ab1 and 75 mg/kg Cry35Ab1) (Marino and Yaroch, 2002; Appendix 2, Section 31). The control diet consisted of the same standard fish diet without the Cry34/35Ab1 ICP. No fish mortality or sublethal effects were observed in any of the control or Cry34/35Ab1 ICP treatment vessels during the study. The 8-day LD_{50} value is greater than 25 mg Cry34/35Ab1 ICP/kg-diet. The predicted Cry34Ab1environmental concentration in a standard EPA farm pond using the senescent plant HEEE expression value for Event DAS-59122-7 is 0.186 mg/L (Poletika, 2003;

Appendix 2, Section 11). Although not directly comparable to a dietary endpoint, this environmental concentration is quite low, indicating low risk to fish. There may not be an actual exposure route for fish to ingest the ICP in nature.

Nontarget Arthropods

Honeybees. There was no effect on mean survival to emergence for honeybee exposed to either 2 mg pollen from a Cry34/35Ab1-expressing event (from an earlier higher expressing event than the subject of this petition) or to 5.6 µg/larvae Cry34/35Ab1 ICP (100X the amount consumed in pollen of the higher expressing event), 3.2 µg/larva Cry34Ab1 protein, or 2.4 µg/larva Cry35Ab1 protein (Maggi, 2001; Appendix 2, Section 25). The LC_{50} is >3.2 ug Cry34Ab1 protein/larva. For the Event DAS-59122-7 pollen HEEE of 75.29 µg/g (Table 41), the equivalent exposure level is 0.07529 µg/mg x 2 mg fed = 0.15 µg/larva.

Green Lacewing. The dietary LC_{50} for green lacewing (*Chrysoperla carnea*) larvae exposed to Cry34/35Ab1 has been investigated in a series of studies with microbially-produced protein administered in a diet of moth eggs (Sindermann *et al.*, 2001; Appendix 2, Section 35). The single limit concentration of 280 µg of Cry34/35Ab1 is 10X the empirical concentration in pollen of a high expressing corn event (16 µg/g Cry34Ab1 + 12 µg/g Cry35Ab1 = 28 µg/g Cry34/35Ab1). The LC_{50} was determined to be greater than 16 µg Cry34Ab1/mL. The target multiple of 10X field exposure for dosing was based on the assumption of a Cry34Ab1 expression level of 16 µg/g in pollen. For Event DAS-59122-7, the HEEE pollen value is 75.29 µg/g (Table 41), resulting in an actual multiple of 16/75.29 x 10 = 2.1X. Field exposure of green lacewing to Cry34/35Ab1 proteins is restricted to larvae feeding on aphids. When the Event DAS-59122-7 Cry34Ab1 HEEE for leaf tissue is multiplied by a conservative estimate of protein transmission efficiency from leaf tissue to aphid, the exposure estimate is 235.03 µg/g x 0.0087 = 2 µg/g (Table 41; Poletika, 2003; Appendix 2, Section 11).

Parasitic Wasp. Parasitic Hymenoptera adults (*Nasonia vitripennis*) were exposed to a single limit concentration of Cry34/35Ab1 in sugar water for up to 10 days. There were no significant differences in mortality between the treatment groups and a sugar water control. The LC_{50} was greater than 160 µg Cry34Ab1/mL of the microbially-derived proteins (Sindermann *et al.*, 2001; Appendix 2, Section 35). The exposure level represents 10X the empirical concentration in pollen of a high expressing corn event. The target multiple for field exposure was based on the assumption of a Cry34Ab1 expression level of 16 µg/g in pollen. For Event DAS-59122-7, the HEEE pollen value is 75.29 µg/g (Table 41), resulting in an actual multiple of 16/75.29 x 10 = 2.1X.

Ladybird Beetle. Adult ladybird beetles (*Hippodamia convergens*) were exposed to a single limit dose concentration (280 µg/mL Cry34/35Ab1 ICP) in sugar water (Bryan, R.L. *et al.*, 2000; Appendix 2, Section 20). The dose concentration was 10X the empirical expression of the Cry34/35Ab1 ICP in pollen (based on the expression of a high-expressing corn event). Ladybird beetle adults fed *ad libitum* over 15 days and were

monitored for mortality and clinical signs of toxicity. Mortality in the control group (22%) was greater than the treatment group (13%), and was not considered related to treatment with the test substances. The LC_{50} for exposure to Cry34Ab1 ICP is >160 µg a.i./mL. The target multiple of 10X for field exposure was based on the assumption of a Cry34Ab1 expression level of 16 µg/g in pollen. For Event DAS-59122-7, the HEEE pollen value is 75.29 µg/g (Table 41), resulting in an actual multiple of 16/75.29 x 10 = 2.1X.

Twelvespotted ladybird beetle (*Coleomegilla maculata)* larvae were fed microbial-expressed protein at a target level of 10X the expected field pollen value in an artificial diet (Higgins, 2003; Appendix 2, Section 10). The actual level of Cry34Ab1 protein in the artificial diet was 900 µg/g, or 12.1X the target multiple. There was no effect observed for the mortality endpoint. However, weight reduction was reported. Twelvespotted ladybird beetle larvae were also fed homozygous Event DAS-59122-7 inbred line pollen mixed with ground corn earworm eggs in a 1:1 weight ratio (Higgins, 2003; Appendix 2, Section 10). This was 1.58X the amount of Cry34Ab1 expected in a commercial hemizygous hybrid. The 1:1 weight ratio resulted in a diet equivalent to one composed of 158%/2 = 79% pollen, or 58.5 µg/g Cry34Ab1 protein. For the endpoints of mortality, delay in development, and weight reduction, there was no effect. Based on the results from the two tests, the LC_{50} is >900 µg/g Cry34Ab1, and the NOEC for weight reduction and delay in development is 58.5 µg/g Cry34Ab1. Mortality is unlikely, because pollen will not contain the elevated level tested using microbial-expressed protein. The HEEE pollen value for Event DAS-59122-7 is 75.29 µg/g (Table 41). Assuming a typical maximum of 50% contribution of pollen in the diet (Poletika, 2003; Appendix 2, Section 11), the actual HEEE exposure is 37.64 µg/g Cry34Ab1. Twelvespotted ladybird beetle prefers to feed on aphids. When the Event DAS-59122-7 Cry34Ab1 HEEE for leaf tissue (Table 41) is multiplied by a conservative estimate of protein transmission efficiency from plant to aphid (Poletika, 2003; Appendix 2, Section 11), the exposure estimate is 235.03 µg/g x 0.0087 = 2 µg/g. Delay in development and weight reduction are not expected with the anticipated level of exposure from a field diet composed of pollen and aphids.

Table 41. Summary of Guideline Hazard Tests for Effect of Cry34/35Ab1 ICP.

Guideline	Study Title	Test Substance	Results
OECD 401	Acute Toxicity—Mouse	Cry35Ab1 microbially-produced protein (TSN102171; 37% pure) Cry34Ab1 microbially-produced protein (TSN102172; 54% pure)	LD_{50} > 2700 mg Cry34Ab1/kg LD_{50} > 1850 mg Cry35Ab1/kg LD_{50} > 2000 mg Cry34/35Ab1/kg
OPPTS 885.4380	Acute Dietary Toxicity LD_{50} – Honeybees	Cry34/35Ab1 pollen from event TC5639; Pollen from nontransgenic isoline (5XH}); Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$NOEC_{pollen}$ = 2 mg/larvae (0.056 µg Cry34/35Ab1 ICP/larvae) $NOEC_{ICP}$ = 20 µg/larvae $NOEC_{Cry34Ab1}$ = 3.2 µg/larvae $NOEC_{Cry35Ab1}$ = 2.4 µg/larvae
OPPTS 885.4340	Non-target Insect – Green Lacewing	Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$LC_{50\ ICP}$ > 280 µg a.i./mL $NOEC_{ICP}$ > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC_{50\ Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL
OPPTS 885.4340	Non-target Insect – Parasitic Hymenoptera	Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$LC_{50\ ICP}$ > 280 µg a.i./mL $NOEC_{ICP}$ > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC_{50\ Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL
OPPTS 885.4340	1)Non-target Insect – Ladybird Beetle (adults; *Hippodamia convergens*)	1) Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	1) $LC_{50\ ICP}$ > 280 µg a.i./mL $NOEC_{ICP}$ > 280 µg a.i./mL $LC_{50\ Cry34Ab1}$ > 160 µg a.i./mL $NOEC_{Cry34Ab1}$ > 160 µg a.i./mL $LC_{50\ Cry35Ab1}$ > 120 µg a.i./mL $NOEC_{Cry35Ab1}$ > 120 µg a.i./mL
	2)Non-target Insect—Ladybird Beetle (larvae; *Coleomegilla maculata*)	2) Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	2) $LC_{50\ ICP}$ > 902 µg a.i./g $NOEC_{ICP}$ > 902 µg a.i./g $LC_{50\ Cry34Ab1}$ > 900 µg a.i./g $NOEC_{Cry34Ab1}$ > 900 µg a.i./g $LC_{50\ Cry35Ab1}$ > 2 µg a.i./g $NOEC_{Cry35Ab1}$ > 2 µg a.i./g
	3) Non-target Insect – Ladybird Beetle (larvae; *Coleomegilla maculata*)	3) Homozygous inbred pollen mixed 1:1 with ground corn earworm eggs	3)Weight gain, delay in development $NOEC_{ICP}$ >58.52 µg a.i./g $NOEC_{Cry34Ab1}$ > 58.5 µg a.i./g $NOEC_{Cry35Ab1}$ > 0.02 µg a.i./g
OECD Proposed	Acute Toxicity - Collembola	Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$LC_{50\ ICP}$ > 12.7 mg a.i./kg diet $NOEC_{ICP}$ > 12.7 mg a.i./kg diet $LC_{50\ Cry34Ab1}$ > 3.2 mg a.i./kg diet $NOEC_{Cry34Ab1}$ > 3.2 mg a.i./kg diet $LC_{50\ Cry35Ab1}$ > 9.5 mg a.i./kg diet $NOEC_{Cry35Ab1}$ > 9.5 mg a.i./kg diet

OECD 202	Acute Dietary Toxicity – *Daphnia magna* *(No pollen)*	Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$EC_{50\ ICP}$ > 100 mg a.i./L $NOEC_{ICP}$ > 100 mg a.i./L $LC_{50\ Cry34Ab1}$ > 57 mg a.i./L $NOEC_{Cry34Ab1}$ > 57 mg a.i./L $LC_{50\ Cry35Ab1}$ > 43 mg a.i./L $NOEC_{Cry35Ab1}$ > 43 mg a.i./L
OECD 207	Acute Toxicity – Earthworm	Cry34Ab1 microbially-produced protein (TSN102171; 54% pure); Cry35Ab1 microbially-produced protein (TSN102172; 37% pure)	$LC_{50\ ICP}$ > 25.4 mg a.i./kg dry soil $NOEC_{ICP}$ > 25.4 mg a.i./kg dry soil $LC_{50\ Cry34Ab1}$ > 6.4 mg a.i./kg dry soil $NOEC_{Cry34Ab1}$ > 6.4 mg a.i./kg dry soil $LC_{50\ Cry35Ab1}$ > 19.0 mg a.i./kg dry soil $NOEC_{Cry35Ab1}$ > 19.0 mg a.i./kg dry soil
OECD 203	Acute Dietary Toxicity – Rainbow Trout	Cry34Ab1 microbially-produced protein (TSN102172; 54% pure); Cry35Ab1 microbially-produced protein (TSN102171; 37% pure)	$LC_{50\ ICP}$ > 100 mg a.i./kg diet $NOEC_{ICP}$ > 100 mg a.i./kg diet $LC_{50\ Cry34Ab1}$ > 25 mg a.i./kg diet $NOEC_{Cry34Ab1}$ > 25 mg a.i./kg diet $LC_{50\ Cry35Ab1}$ > 75 mg a.i./kg diet $NOEC_{Cry35Ab1}$ > 75 mg a.i./kg diet
OPP 71-2 OECD 205	Protein Equivalency - Broilers	Cry34/35Ab1 grain from event 15344	Mortality, weight gain, feed efficiency, carcass yields $LC_{50\ ICP}$ > 25.1 ng a.i./mg diet $NOEC_{ICP}$ > 25.1 ng a.i./mg diet $LC_{50\ Cry34Ab1}$ > 23 ng a.i./mg diet $NOEC_{Cry34Ab1}$ > 23 ng a.i./mg diet $LC_{50\ Cry35Ab1}$ > 2.1 ng a.i./mg diet $NOEC_{Cry35Ab1}$ > 2.1 ng a.i./mg diet

VI.C. ENVIRONMENTAL EXPOSURE

Non-target organisms may be exposed to Cry34/35Ab1 ICP expressed in event DAS-59122-7 through either direct or indirect routes. Exposure estimates for organisms directly feeding on corn tissues expressing Cry34/35Ab1 protein are based on the high-end expression for the relevant plant tissue to which a non-target organism of concern may be exposed through direct ingestion. High-end exposure estimates (HEEE) represent the 90% upper bound of the reported expression (USEPA, 1997). Indirect exposures represent inadvertent exposures to Cry34/35Ab1 ICP through soil, water, pollen on host plant tissues, or multitrophic interactions. These exposures are expressed as Estimated Environmental Concentrations (EEC) and are conservatively calculated using high-end estimates for input parameters.

VI.C.1. EXPOSURE ROUTES

Direct feeding on plants or plant parts constitutes the primary route of exposure of organisms to Cry34/35Ab1 protein expressed in event DAS-59122-7. Plant parts subject to feeding are predominantly leaves, roots, stems and pollen. Organisms directly feeding on corn as a primary food source within agroecosystems would be characterized as plant pests and are not germane to this assessment. Organisms incidentally exposed to plant residues or organisms consuming corn plants or plant parts as an occasional or supplementary food source are considered non-target organisms of concern in this exposure assessment. Secondary exposure to protein residues by tritrophic interactions may occur for predators or parasites of plant-feeding organisms. Residues occurring in soil or water matrices may constitute an additional secondary route of exposure to Cry34/35Ab1 protein. The no-effect levels for non-target ecotoxicity tests show adequate margins of safety relative to conservatively projected environmental exposure concentrations (see section VI.C.), and these observations are supported in field monitoring for species abundance (Higgins, 2003; Appendix 2, Section 12). Thus, the exposure routes postulated here are relevant only to exposure and risk characterization for potentially sensitive taxa of Lepidoptera and Coleoptera.

VI.C.2. ENVIRONMENTAL FATE OF CRY34/35AB1 ICP INCORPORATED INTO SOIL

The time-dependent loss in bioavailability of Cry34/35Ab1 ICP following incorporation into a typical corn-growing soil was determined under laboratory conditions (Herman, 2000; Appendix 2, Section 26). Test treatments consisted of Cry34Ab1 and Cry35Ab1 ICP, mixed, and applied to soil as well as untreated soil (as a negative control). Montmorenci soil, obtained from Benton County, IN, was fortified with an aqueous mixture of the ICP (61.7 mg Cry34Ab1 powder (TSN102172) + 90 mg Cry35Ab1 powder/mL (TSN102171)) to achieve a target concentration of 5 mg ai/gm soil for each

protein. The volume of water applied along with the test materials was calculated to bring the soil moisture to 75% of the 1/3 bar water holding capacity (WHC). Samples were held at 25°C under low pressure oxygen and were subsequently removed at 0, 1, 3, 5, 7, 14 and 28 days following fortification and immediately frozen at – 80°C until prepared for bioassay. For each bioassay, treatments were suspended in 10 ml of 0.2% agar solution. The bioassay measured growth inhibition of *Diabrotica undecimpunctata howardii* (southern corn rootworm) neonate larvae following exposure to a series of doses in a diet incorporation assay. For each treatment/exposure length combination, the arcsine of the growth inhibition was regressed against the base-10 logarithm of the concentration (ug ai/cm^2 of insect diet) and the resulting line was used to predict the concentration that inhibits growth by 50% (GI_{50}) and 95% confidence limits around this estimate. A half-life based on a first order degradation was calculated by regressing the natural logarithm of the reciprocal GI_{50} against time and substituting the slope of the regression in the following formula: $T_{1/2}$ = -0.693/slope. The GI_{50} increases as the ICP degrades so the inverse GI_{50} is used to index degradation in the aforementioned calculations. The GI_{50} calculations from the 14-day and 28-day incubation periods were excluded from the half-life calculations because these data did not fit a first order decay. The first-order decay is a conservative approach for calculating the half-life. The complete data set was modeled in a separate analysis (Herman *et al*., 2002).

The results of this study are given in Table 42. The southern corn rootworm GI_{50} calculations (concentration estimated to reduce growth by 50%) showed that there was no statistical difference between the potency of the spiked control and the 0-day incubation period indicating that signficant degradation of ICP did not occur once soil samples were frozen. The half-life of the microbial ICP in soil, based on a first order degradation, was calculated to be 3.2 days using the 0-day through 7-day results. This is a rapid decay rate and is consistent with that reported for other *Bt* proteins (Shanahan & Stauffer, USDA, 2000; Sims & Holden, 1996; Herman *et al.*, 2002).

Table 42. Southern corn rootworm GI_{50} calculations for incubation periods of the microbial Cry34/35Ab1 ICP in soil. Montmorenci soil, obtained from Benton County, IN, was fortified with an aqueous mixture of the ICP (61.7 mg Cry34Ab1 powder (TSN102172) + 90 mg Cry35Ab1 powder/mL (TSN102171)) to achieve a target concentration of 5 mg ai/gm soil for each protein.

	GI_{50} (95% Confidence Limits) in ug ai ICP/cm^3
Treatment	
Spiked Control	2.5 (0.77 - 7.9)
0-Day	2.3 (1.0 - 5.3)
1-Day	3.0 (0.69 - 13)
3-Day	6.0 (2.0 - 18)
7-Day	10 (2.8 - 38)
14-Day	14 (6.6 - 31)
28-Day	18 (3.2 - 103)

VI.C.3. ESTIMATED LEVELS OF ENVIRONMENTAL EXPOSURE

Evaluation of protein expression levels and routes of exposure allows for development of estimated levels of exposure conservatively projected to occur in the environment.

HIGH END EXPOSURE ESTIMATES (HEEE)

High-end exposure estimates (HEEE) are shown in Table 43 for those tissues relevant to conservatively estimating exposure concentrations by an identified route of exposure.

Table 43. High end exposure estimates (HEEE) for expression of Cry34/35Ab1 ICP. The HEEE are based upon expression values obtained from corn plants not sprayed with glufosinate-ammonium and plants sprayed with glufosinate-ammonium.

	Cry34Ab1				Cry35Ab1				Sum of Cry34 and Cry35
Tissue (Growth Stage)	**Mean[a]**	**Std. Dev.**	**HEEE[b]**	**n**	**Mean**	**Std. Dev.**	**HEEE**	**n**	**HEEE**
	µg g^{-1} tissue dry weight				**µg g^{-1} tissue dry weight**				**µg g^{-1} tissue dry weight**
Leaf (V9)	45.93	7.56	47.19	60	36.27	9.07	37.79	60	84.98
Whole Plant (V9)	39.41	22.76	48.31	12	7.75	1.92	8.50	12	56.81
Root (V9)	38.48	8.72	40.37	36	8.05	2.80	8.65	36	49.02
Pollen (R1)	74.27	6.09	75.29	60	0.02	0.03	0.03	60	75.32
Stalk (R1)	31.03	4.62	31.81	60	8.64	2.54	9.06	60	40.87
Root (R1)	40.58	10.43	42.32	60	5.24	1.58	5.50	60	47.82
Leaf (R1)	76.24	12.57	78.34	60	54.51	14.30	56.91	60	135.25
Whole Plant (R1)	46.48	14.23	52.05	12	12.66	4.27	14.34	12	66.38
Forage (R4)	46.58	24.23	56.06	12	13.25	2.89	14.38	12	70.44
Root (R4)	57.98	22.00	62.75	36	3.70	1.02	3.93	36	66.68
Leaf (R4)	226.44	39.52	235.03	36	83.01	18.39	87.00	36	322.03
Grain (R6, Harvest)	55.39	18.63	58.51	60	0.95	0.31	1.01	60	59.52
Whole Plant, Including Grain (R6, Senescence)	86.30	18.01	93.35	12	15.38	2.93	16.52	12	109.87
Whole Plant, Excluding Grain (R6, Senescence)			109.03 [c]						132.53 [d]
Root (R6, Senescence)	54.04	20.83	58.56	36	3.33	2.18	3.80	36	62.37
Leaf (R6, Senescence)	149.52	81.93	167.31	36	53.20	23.26	58.25	36	225.57

[a] Data are from Essner, 2003 and calculations reported in Poletika, 2003. Non-detects were taken as ½ the limit of detection (0.01 µg/g).

[b] High-end exposure estimate = mean + ($t_{0.1,\ upper\ tail,\ n-1}$ x std. dev.) / $n^{1/2}$.

[c] Grain g dw/A = ((235 bu/A – 235 bu/A x (1-0.85 dry weight)) x 56 lb/bu) x 1 g/0.002205 lb = 5073016
Stover g dw/A = Grain g dw/A x 1/0.45 = 11273369
Protein in stover µg/g dw = (Protein in Whole Plant, Including Grain (R6, Senescence) x Sum of Grain+Stover g dw/A Protein in Grain (R6, Harvest) x Grain g dw/A) x 1/Stover g dw/A = (93.35 x 16346384 – 58.51 x 5073016) x 1/11273369 = 109.03

[d](109.87 x 16346384 – 59.52 x 5073016) x 1/11273369 = 132.53

Source of yield and lb/bu: Martin et al., 1976; source of dry weight percentage: Morrison, 1961.

Soil and Water. The HEEE in senescent plants can be used as a worst-case exposure to evaluate risk of Event DAS-59122-7 Cry34Ab1 protein residues to detritivores feeding directly on plant residue. This value is 109.03 µg/g (Table 43). Earthworms ingest soil particles, and collembola feed on plant material incorporated into soil, so a more realistic estimated environmental concentration (EEC) assumes uniform mixing of the plant residue at a soil depth of 6 inches. Using plant residue load estimates in soil from the

toxicity studies (Teixeira, 2001; Bryan et al., 2000; Appendix 2, Sections 34 and 20), a worst-case EEC calculation is as follows:

Protein in soil = 25,000 plants/A x 1 kg/plant x 109030 µg protein/kg plant / 908,000 kg soil/A = 3002 µg/kg.

Pollen can be transported by wind over short distances, and proteins released within the field can become entrained in surface runoff and eroded sediment. If a water body is nearby, then deposition of pollen and free protein is possible, resulting in exposure to organisms in the water column. The worst-case water-column EEC results from a no-till scenario in which all of the ICP remaining in senescent plant tissue on the surface is released and becomes immediately available for runoff (Poletika, 2003; Appendix 2, Section 11).

Assuming a standard USEPA farm pond of dimension 63.61 m x 63.61 m x 2 m (USEPA, 2001) is adjacent to a cornfield planted to Event DAS-59122-7 at a plant population of 25,000 plants per acre and that these plants express Cry34Ab1 protein at 109.03 µg/g (Table 43), the protein available for runoff is:

Protein on soil = 25,000 plants/A x 1 kg/plant x 109030 µg/kg x g/10^6 µg x lb/453.6 g = 6.0 lb/A.

A generic EEC is predicted by the USEPA program GENEEC (USEPA, 2001) using a 6.0 lb/A "application rate" and conservative values for environmental fate properties (Poletika, 2003; Appendix 2, Section 11). The peak instantaneous Cry34Ab1 EEC is 185.89 µg/L

Pollen. A corn pollen EEC near commercial fields is not calculated because no terrestrial nontarget organism outside the planted field is differentiated from those occurring inside, where a high level of pollen is available as a food source during pollen shed. The literature suggests that the only species frequenting cornfields considered to be at risk is the twelvespotted ladybird beetle, *Coleomegilla maculata*, due to its habit of feeding on pollen (Poletika, 2003; Appendix 2, Section 11). Toxicity testing at an elevated dose higher than that expected in a diet containing 50% pollen demonstrates safety in this species (Higgins, 2003; Appendix 2, Section 10). Therefore, no in-field pollen EEC is necessary.

Phytophagous Insects. The magnitude of secondary exposure resulting from consumption of herbivorous prey containing residues of Cry34/35Ab1 protein is dependent on the expression levels in plant parts eaten and the efficiency of protein transmission from plant to prey. Although there are no data for Cry34/35Ab1, transmission efficiencies available for Cry1Ab protein in seven different species can be used to estimate generic transmission of Bt insecticidal proteins (Raps et al., 2001; Head et al., 2001). To be conservative, the largest reported Cry1Ab transmission efficiencies for aphids, 0.0087, and noctuid larvae, 0.11, (Raps et al., 2001) are applied to estimates of secondary exposure in predators and parasitoids. An appropriate worst-case Cry34Ab1 HEEE calculated for Event DAS-59122-7 is the maximum HEEE in Table 43

for all tissues (leaf, R4) multiplied by the maximum known transmission factors: 235.03 μg/g x 0.0087 = 2.0 μg/g for aphids and 235.03 μg/g x 0.11 = 26 μg/g for noctuid larvae.

Beneficial Insect Considerations. Important groups of predators and parasitoids in field corn include ladybird beetles (Coleoptera, Coccinellidae), ground beetles (Coleoptera, Carabidae), rove beetles (Coleoptera, Staphylinidae), tachinid flies (Diptera, Tachinidae), braconid wasps (Hymenoptera, Braconidae), ichneumonid wasps (Hymenoptera, Ichneumonidae), flower bugs and minute pirate bugs (Hetroptera, Anthocoridae), green lacewings (Neuroptera, Chrysopidae), and brown lacewings (Neuroptera, Hemerobiidae), (Steffy et al., 1999). Risks of adverse impacts on these beneficial insects were considered in Poletika (2003; Appendix 2, Section 11). Significant biological activity of the Cry34/35Ab1 ICP is restricted to larvae of chrysomelid beetles (Coleoptera, Chrysomelidae), which are pests in field corn. Activity in other coleopterans is not observed. Toxicity testing in green lacewing, parasitic hymenoptera, and convergent ladybird beetle at levels greater than those expected in the field fails to identify effects of concern. Of all the listed beneficial groups, only the Coccinelidae has a species (*Coleomegilla maculata*) known to feed directly on corn tissue (pollen) in any significant amount. Toxicity testing indicates this species is not sensitive to Cry34/35Ab1 ICP. There is negligible risk from direct feeding on corn tissue. Secondary exposure is possible from protein residues transmitted from phytophagous prey to predators or parasitoids. However, transmission is very inefficient, and the exposure level is quite low. Secondary exposure poses little risk to beneficial insects. Field monitoring of hybrid lines expressing Cry34/35Ab1 ICP finds no population or community level effects, confirming the conclusion that beneficial insects are not at risk.

Endangered Species Considerations. B. thuringiensis subspecies are differentiated by their insecticidal activity. Generally, only insect species within a given order (*Lepidoptera, Coleoptera, Diptera,* and *Hymenoptera*) are susceptible to a given insecticidal *B.t.* δ-endotoxin protein. Therefore, insect susceptibility results provide general information about the specificity of δ-endotoxin(s) expressed by particular *B. thuringiensis* strains. In the case of *B. thuringiensis* PS149B1strains, the greatest activity is shown for the order *Diabrotica.* Toxicological studies on non-target beneficial insect species using Cry34/35Ab1 corn pollen or microbially-expressed protein support selectively within Coleoptera, given the margins of safety shown for representative species from other orders (*Hymenoptera, Diptera, and Lepidoptera*).

Since Cry34Ab1/Cry35Ab1 targets the western corn rootworm *Diabrotica virgifera virgifera* LeConte (a beetle), an individual evaluation of each beetle species on the endangered/threatened species list was conducted (Higgins, 2003; Appendix 2, Section 15). Almost all endangered beetle species occur outside of row crop systems with the exception of the American burying beetle, which depends on carrion buried in the ground for survival. Based on the habitat and life cycle information, there is little probability that Cry34Ab1/Cry35Ab1 hybrids pose a threat to the American burying beetle or other endangered/threatened beetle species. Additionally, the specificity of activity observed in laboratory bioassays indicates that Cry34Ab1/Cry35Ab1 will not likely have an impact on endangered insects occurring in other insect orders.

Table 44 lists the 16 Coleopteran insect species either classified as endangered or threatened by the U.S. Fish and Wildlife Service. Figure 72 highlights the counties where endangered beetle species are known to occur in relationship to the amount of corn production. Only one endangered beetle species exists in areas of high corn cultivation (the American burying beetle).

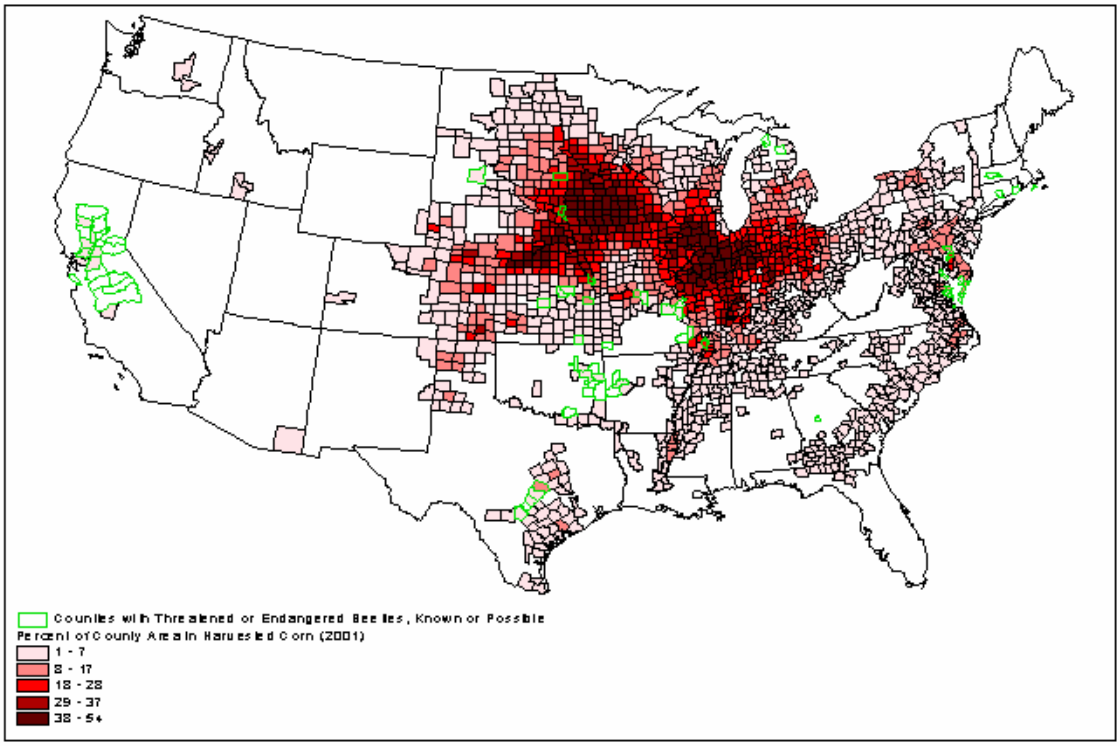
The American burying beetle (*Nicrophorus americanus*) is the largest carrion beetle in North America and was formerly widespread throughout temperate eastern North America. Today it is found in limited areas in Rhode Island and the portions of the Great Plains into Arkansas and Georgia. Biology of the American burying beetle is similar to other species in the genus. Adults are nocturnal, generally active from late April though September, feed on carrion and sometimes prey on other arthropods. Larvae feed exclusively on the buried carrion provided by their parents.

The American burying beetle's habitat is variable and often includes deciduous forest, grassland, and agricultural areas (USF&WS 1991). Availability of carrion in a given area is more important to American burying beetle occurrence than the vegetation or soil structure (US Fish &Wildlife Service, 1991). Research continues into possible reasons for population decline of the American burying beetle, which was well on its way by the early 1920's (US Fish &Wildlife Service, 1991). A prevailing theory is that changes in land use and the resulting fragmentation of the American burying beetle habitat lead to a reduction in optimum reproductive carrion resources. Extinction of land birds such as the passenger pigeon and the greater prairie chicken (which were in the favored weight range for the American burying beetle) may have played a role in population reduction (US Fish &Wildlife Service, 1991). Other possible influences to American burying beetle populations include pesticide use, competition by other scavengers and artificial light that can disrupt nocturnally active animals. Considering that both larvae and adult insects feed exclusively on carrion with some limited adult predation, it appears that even if American burying beetles did occur in proximity to *Bt* corn fields, there would be little chance of exposure to *Bt* protein due to their feeding habits.

Table 44. Coleopteran insect species either classified as endangered or threatened by the U.S. Fish and Wildlife Service (E=endangered, T=threatened).

Order	Species	Status
Coleoptera	American burying beetle (*Nicrophorus americanus*)	E
Coleoptera	Coffin cave mold beetle (*Batrisodes texanus*)	E
Coleoptera	Comal Springs dryopid beetle (*Stygoparnus comalensis*)	E
Coleoptera	Comal Springs riffle beetle (*Heterelmis comalensis*)	E
Coleoptera	Delta green ground beetle (*Elaphrus viridis*)	T
Coleoptera	Helotes mold beetle (*Batrisodes venyivi*)	E
Coleoptera	Hungerford's crawling water beetle (*Brychius hungerfordi*)	E
Coleoptera	Kretschmarr cave mold beetle (*Texamaurops reddelli*)	E
Coleoptera	Mount hermon june beetle (*Polyphylla barbata*)	E
Coleoptera	Northeastern beach tiger beetle (*Cicindela dorsalis dorsalis*)	T
Coleoptera	Ohlone tiger beetle (*Cicindela ohlone*)	E
Coleoptera	Puritan tiger beetle (*Cicindela puritana*)	T
Coleoptera	Rhadine exilis (NCN)	E
Coleoptera	Rhadine infernalis (NCN)	E
Coleoptera	Tooth cave ground beetle (*Rhadine persephone*)	E
Coleoptera	Valley elderberry longhorn beetle (*Desmocerus californicus dimorphus*)	T

Figure 72. Distribution of endangered beetle species habitat relative to corn acreage in the continental United States.



Cry34Ab1 and Cry35Ab1 are coleopteran active proteins, and toxicological data produced by the registrants show the relative insensitivity of a range of insects from non-coleopteran orders to the Cry34Ab1/Cry35Ab1 proteins (Section VI.B,Table 45). The No Effect Limit Dose for representatives of other insect groups greatly exceeds the values for Cry34/35Ab1 ICP (the LC_{50} for the target pests northern corn rootworm and western corn rootworm are 5.56 ug/cm^2 and 44.50 ug/cm^2 respectively), indicate that corn hybrids containing the ICP will not likely cause detrimental effects to the non-coleopteran insects on the endangered/threatened species list (Table 45). Additionally, there are geographic and habitat considerations that negate any exposure risk. The dipteran endangered species *Rhaphiomidas terminatus abdominalis* (the Delhi sands flower-loving fly) and the orthopteran endangered species Trimerotropis infantilis (Zayante band-winged grasshopper) both occur in California. The threatened hemipteran species *Ambrysus amargosus* (the Ash Meadows Naucorid) occurs in Nevada. Since both these states occur west of the Rocky Mountains where western corn rootworm do not occur, testing and planting of CRW-resistant hybrids is not expected to take place in habitats containing

these endangered/threatened species. The endangered Odonata species Somatochlora hineana (Hine's emerald dragonfly), occurs in the Midwest (specifically Ohio, Illinois and Wisconsin) but would unlikely be affected by *Bt* corn since the larval stage is aquatic and adults are predacious. Many of the lepidopteran species on the endangered/threatened species list are found in dune, meadow/prairie or open forest habitats and are not closely associated with row crop production often times due to the specificity of the habitat of their host plants.

Table 45. Insect species (other than those occurring in the Order Coleoptera) either classified as endangered or threatened by the U.S. Fish and Wildlife Service (E=endangered, T=threatened).

Order	Species	Status
Diptera	Delhi sands flower-loving fly (*Rhaphiomidas terminatus abdominalis*)	E
Hemiptera	Ash Meadows Naucorid (*Ambrysus amargosus*)	T
Lepidoptera	Bay checkerspot butterfly (*Euphydryas editha bayensis*)	T
Lepidoptera	Behren's silverspt butterfly (*Speyeria zerene behrensii*)	E
Lepidoptera	Blackburn's sphinx moth (*Manduca blackburni*)	E
Lepidoptera	Callippe silverspot butterfly (*Speyeria callippe callippe*)	E
Lepidoptera	Carson wandering skipper (*Pseudocopaeodes eunus obscurus*)	E
Lepidoptera	El Segundo blue butterfly (*Euphilotes battoides allyni*)	E
Lepidoptera	Fender's blue butterfly (*Icaricia icarioides fenderi*)	E
Lepidoptera	Karner blue butterfly (*Lycaeides melissa samuelis*)	E
Lepidoptera	Kern Primrose sphinx moth (*Euproserpinus euterpe*)	T
Lepidoptera	Laguna Mountains skipper (*Pyrgus ruralis langunae*)	E
Lepidoptera	Lange's metalmark butterfly (*Apodemia mormo langei*)	E
Lepidoptera	Lotis blue butterfly (*Lycaeides argyrognomon lotis*)	E
Lepidoptera	Mission blue butterfly (*Icaricia icarioides missionensis*)	E
Lepidoptera	Mitchell's satyr butterfly (*Neonympha mitchellii michellii*)	E
Lepidoptera	Myrtle's silverspot butterfly (*Speyeria zerene myrtleae*)	E
Lepidoptera	Oregon silverspot butterfly (*Speyeria zerene hippolyta*)	T
Lepidoptera	Palos Verdes blue butterfly (*Glaucopsyche lygdamus palosverdesensis*)	E
Lepidoptera	Pawnee montane skipper (*Hesperia leonardus montana*)	T
Lepidoptera	Quino checkerspot butterfly (*Euphydryas editha quino*)	E
Lepidoptera	St. Francis' satyr butterfly (*Neonympha mitchellii francisci*)	E
Lepidoptera	San Bruno elfin butterfly (*Callophrys mossii bayensis*)	E
Lepidoptera	Schaus swallowtail butterfly (*Heraclides aristodemus ponceanus*)	E
Lepidoptera	Smith's blue butterfly (*Euphilotes enoptes smithi*)	E
Lepidoptera	Uncompahgre fritillary butterfly (*Boloria acrocnema*)	E
Odonata	Hine's emerald dragonfly (*Somatochlora hineana*)	E
Orthoptera	Zayante band-winged grasshopper (*Trimerotropis infantilis*)	E

Field Census Study. A field experiment was designed to evaluate the impacts of corn rootworm control strategies, including the cultivation of Cry34/35Ab1 corn, on non-target arthropod populations (Higgins, 2003; Appendix 2, Section 12). The study was conducted at two locations in the central Corn Belt of the US (York, NE and Johnston, IA) over two growing seasons in 2001-2002. Each location consisted of two replications per treatment in a randomized complete block design. Each replicate consisted of five treatements: 2 treatments of Cry34/35Ab1 corn hybrids (events 15344 and 5639 which

express the Cry35Ab1 protein at higher levels than DAS-59122-7), a non-*B.t.* control hybrid treated with a soil insecticide, a non-*B.t.* control hybrid treated with a foliar insecticide and an untreated non-*B.t.* control hybrid, planted in plots of approximately 2800 ft^2. Plots and treatments remained fixed for both growing seasons of the study. Four methods of sampling were employed throughout both growing seasons: visual observations, sticky traps, pitfall traps and soil core samples. Data were collected on individual families, however, it was often more meaningful and statistically powerful to group some of the families for analysis. Community level analysis was used to look for a general community reponse to the treatments, separately in 2001 and 2002. Every taxon (group) that was observed in the field during the study was included in the community level analysis. For each key indicator species, analysis of variance (ANOVA) based on a mixed linear model was carried out. These ANOVAs were used to compare abundance in the treatment plots to abundance in control plots and were intended to detect differences that may occur in individual taxa (taxa groups) whose response may differ from the primary community response.

Analysis of the pitfall trap data showed no significant results in the overall community analysis in 2001, but did in 2002. In 2001, analysis of each sampling stage separately showed significant differences at R2 and R5. The largest effect shown by this analysis appears to be an increase in abundance at R2 and R5 of some species (in particular, collembola) in the control-foliar treatment when compared with the untreated control. These effects coincided with and may be explained by the application of a foliar insecticide, as changes in the collembola population may be a result of a change in the natural enemy complex. In 2002, analysis of each sampling stage separately showed significant differences at V4 and R2. At V4, the largest effect appears to be an increase in abundance under the control-soil treatment relative to the untreated control in some species (primarily collembola). As was true in the 2001 PRC analysis, in 2002 there appears to be an increase in abundance under the control-foliar treatment when compared with the untreated control at R2 (and R5, though not statistically significant at R5 in 2002) in some species (primarily collembola).

For pitfall traps, the following key taxa were analyzed using ANOVA: elongate collembola, globular collembola, orbatid mites, centipedes, millipedes, ground beetles (adult and larvae), rove beetles (adult and larvae) and spiders. For elongate collembola, a significant treatment main effect was observed, although treatment differences were dependent on sampling stage and environment, as indicated by a significant Location*Year*Stage*Treatment interaction. There were 7 stages in which a treatment showed a significant difference from the untreated control in the same direction in more than one environment. Of those 7 stages, 2 involved a Cry34Ab1/Cry35Ab1 hybrid treatment. At 50% pollen shed, event 5639 showed a slight decrease in abundance compared to the untreated control, and at post harvest, event 5639 showed at slight increase in abundance compared to the untreated control. Usually the differences observed were small and are not likely meaningful. For the remaining key taxa, there were no significant differences between a treatment and the untreated control (no ANOVA was performed on ground beetle or rove beetle larvae due to low abundance in all treatment groups).

The community level analyses of the soil samples showed no statistically significant differences between any treatment with the untreated control at any sampling stage or over all sampling stages in either year of the study.

For soil samples, the following key taxa were analyzed using ANOVA: elongate collembola, globular collembola, orbatid mites, centipedes, millipedes, ground beetles (adult and larvae), rove beetles (adult and larvae) and spiders. No significant Cry34Ab1/Cry35Ab1 treatment effects were observed when compared to the untreated control for elongate collembola, globular collembola, orbatid mites, and centipedes. No ANOVA was performed on ground beetle adults or larvae, rove beetle adults or larvae, millipedes or spiders due to low abundance in all treatment groups.

Analysis of the sticky trap data showed no significant results in the overall community level analysis in 2001 or 2002. In 2001, analysis of each sampling stage separately showed significant differences at VT. Other than for wCRW adults, the analysis indicated a possible increase in abundance relative to the untreated control at VT for the two events and a possible decrease in abundance under the soil and foliar treatments. In 2002, no significant differences were found in the community level analysis of any sampling stage.

For sticky traps, the following key taxa were analyzed using ANOVA: Ladybird beetle adults, insidious flower bugs (Orius), aphids, fairy flies (Mymaridae), leaf hoppers, long-legged flies (Dolichopodidae), rove beetles and thrips. No significant Cry34Ab1/Cry35Ab1 treatment effects were observed for ladybird beetle adults, Orius, fairy flies, long-legged flies, rove beetles, thrips and leafhoppers when compared to the untreated control. An overall treatment effect was detected for leafhoppers showing a decrease in abundance in the control – foliar treatment compared to the untreated control when averaged over all environments and sampling stages, however, this difference was very small. For aphids, there was a statistically significant Year*Treatment*Stage interaction observed. Of the significant differences that involved a Cry34Ab1/Cry35Ab1 event, two stages showed an increase in abundance compared to the control (at V6 in 2002 and VT in 2001), and one showed a decrease in abundance (at R5, 2001), but this decrease was less than one insect.

A significant difference was detected between the five treatments in the community level analysis of the visual observations in 2001, but was not significant in 2002. Analysis of each sampling stage separately showed significant differences at 50% shed and R2 in 2001. Based on the PRC graph, the largest effect is a decrease in abundance under the control-foliar treatment relative to the untreated control at 50% shed. In 2002, analysis of each sampling stage separately showed significant differences at 50% shed. Consistent with 2001 results, the largest effect at 50% shed corresponds to a decrease in abundance under the control-foliar treatment relative to the untreated control. These effects coincided with and may be explained by the application of a foliar insecticide.

For visual observations, the following key taxa were analyzed using ANOVA: lacewing eggs, lacewing larvae, ladybeetle eggs, ladybeetle larvae and ladybeetle adults. For lacewing eggs, no significant overall treatment effects were detected, but there was a significant Year*Location*Treatment interaction. When investigated, the only treatments that showed a significant difference from the untreated control were as follows: a small increase in abundance under event 15344 in two environments averaged over stages, and a small decrease in abundance in one environment under the control-foliar treatment. Abundance of lacewing larvae was low in all treatment groups, so no ANOVA could be preformed. For ladybeetle adults, a significant overall treatment effect was detected, showing a slight increase in the abundance in Cry34Ab1/Cry35Ab1 event 15344 compared to the untreated control. A Year*Treatment interaction was also detected showing a decrease in abundance in the control-foliar treatment in 2001 when compared to the untreated control when averaged over all sampling stages and locations. For ladybird beetle larvae, a significant treatment effect was detected. There was a decrease in abundance under the control-foliar treatment when considered over stages VT-R5 (all four environments) and this difference was greatest at 50% pollen shed. There were no treatment differences detected for ladybird beetle eggs.

In conclusion, the Cry34/Cry35Ab1 hybrids (containing events 15344 and 5639) exhibited no signs of detrimental effects on non-target organisms. The two primary effects observed in the study were an overall abundance decrease (as measured by visual observation) at the 50% pollen shed sampling date in the foliar insecticide treated plots compared to the untreated control plots and an increase in collembola abundance (as measured in pitfall traps) at the R2 and R5 sampling stages of the foliar insecticide treated plots compared to the untreated control plots. Other isolated differences did exist (involving other treatments including Cry34Ab1/Cry35Ab1 events), but these differences were sporadic and usually small. While the two Cry34/Cry35Ab1 events represented will not be commercially available, they represent equal or greater Cry34/Cry35Ab1 expression levels when compared to the commercial event DAS-59122-7. These findings, therefore, are directionally appropriate to DAS-59122-7 and confirm the conclusion of negligible risk arising from laboratory studies and assessments.

VI.D. POTENTIAL ADVERSE EFFECTS ON HUMAN AND ANIMAL HEALTH

Plant compositional analysis (section V.D.) and toxicity tests (section VI.B.) show no adverse consequences of event DAS-59122-7 or the Cry34/35Ab1 ICP it expresses on human or animal health.

VI.D.1. HUMAN HEALTH RISK

Dietary exposure

The average expression of Cry34Ab1 and Cry35Ab1 in plant tissues of line 59122 is 55.39 ng/mg and 0.95 ng/mg dry weight, respectively (based on unsprayed data and

sprayed data from glufosinate-ammonium treatement from the Chile experiment). The actual exposure to Cry34/35Ab1 ICP in the diet is expected to be much lower due to: 1) protein degradation during transport and storage; 2) grain containing the ICP will be mixed with other grain not containing the ICP; 3) reductions in protein concentrations will occur during processing to produce high fructose corn syrup and vegetable oils (commodities that contain negligible levels of protein) (Layton, 2003; Attachement 1).

A simple way to characterize dietary risk is to calculate the amount of a food that would have to be eaten to expose a person to the same level of protein that was utilized in toxicology studies of the protein. The estimated amount of food is then evaluated in terms of how feasible it would be to eat that amount of food in one day. For example, Cry34Ab1 expression in event DAS-59122-7 grain is approximately 55 ng/mg dry weight. This means that one milligram of grain contains 0.000055 mg of protein. Mice were dosed using 2700 mg of microbially produced protein per kilogram body weight. To receive an equivalent dose of Cry34Ab1 protein in grain, each mouse would have had to eat 48.7 kg of grain per kg body weight. Similar estimates can be made as well for Cry35Ab1. Following these assumptions, one can calculate the amount of raw grain that would have to be eaten by an infant, child, or an adult to match the protein levels used in the mouse toxicity studies.

	Cry34Ab1 Alone	Cry35Ab1 Alone	Cry34Ab1 Combination	Cry35Ab1 Combination
Expression (ng/mg)	55.39	0.95	55.39	0.95
Tox Test Dose (mg/kg bw)	2700	1850	482	1520
Amount (kg/day) of grain needed to be consumed to match dose in toxicology tests:				
Child (10 kg)	487	19474	44	8000
Adult (60 kg)	2925	116842	522	96000

Based on these simplistic worst-case calculations, it is obvious that there is a clear margin of safety for these proteins. The actual margin of safety will be much greater when the effects of factors such as market share and processing are taken into account.

VI.E. WEEDINESS OF *B.T.* CRY34/35AB1 CORN LINE 59122

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

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

The introduction of the trait for *Diabrotica* resistance should not confer a weediness trait to corn as protection from specific *Diabrotica* feeding would not increase the fitness capabilities of this corn line. Similarly, the trait for resistance to glufosinate-ammonium herbicides has been used widely in plant breeding as a selective marker. The addition of this phenotypic trait to the *B.t.* PS149B1 corn line described in this petition is not expected to increase fitness. As noted above, the survival of volunteer corn plants in the agricultural fields is easily controlled by manual or chemical means.

There are no wild, weedy relatives of *Zea mays* known to exist in the United States. Therefore, outcrossing of the *cry*34/35Ab1 genes or *pat* gene does not pose a plant pest risk due to the enhancement of weediness of wild relatives of corn.

VI.F. PROPOSED INSECT RESISTANCE MANAGEMENT PLAN

The cost of discovering and developing transgenic traits conferring high levels of resistance to pests is very high. Dow AgroSciences and Pioneer intend to implement measures that protect the durability of the trait by reducing the rate at which the pests adapt. This will help ensure that the benefits of the technology to the growers and to the environment are sustainable for the long term. DAS/PHI will implement a product durability plan consisting of a set of scientifically-based practical measures that are intended to achieve this aim.

Resistance management for Bt corn has become well established since the first Bt corn was commercially grown in 1996. Corn growers, consultants, and entomologists, as well as regulatory authorities, all agree on the need to preserve the benefit of the technology, and that the best tool is the planting and managing of refugia consisting of non-Bt corn. Several refuge options are available to growers, and have proven to be effective at maintaining pest susceptibility in the face of extensive use of Bt crops.

Cry34/35Ab1 rootworm-protected corn has high efficacy against the key target pests. Field and laboratory research, has established that Cry34/35Ab1 rootworm-protected corn kills more than 99.8% of wCRW larvae, and at least 95% of NCR larvae, while having no effect on adults. Published computer simulation models indicate that while a 10% refuge is likely to be adequate to manage resistance in wCRW, a 20% refuge is appropriate for NCR. Therefore, a 20% refuge is recommended to protect the durability of Cry34/35 rootworm-protected corn. This refuge should be planted in the same field as the Cry34/35 rootworm-protected corn, or in an adjacent field. In-field options include single blocks or strips (minimum width of 2 rows); seed mixtures are not likely to be effective. The refuge corn can be protected from rootworm damage using banded soil

insecticides or insecticide-treated seed. If the Cry34/35Ab1 rootworm-protected corn is planted on acreage that was in corn the previous year, then the refuge corn should also be planted on acreage that was in corn the previous year to ensure adequate production of susceptible beetles in the refuge. Management of pests other than rootworms can follow local integrated pest management guidelines; refuge and Cry34/35Ab1 rootworm-protected corn can be managed independently. Computer models predict that this product durability plan will protect the durability of Cry34/35Ab1 rootworm-protected corn.

Several tools are currently available and effective for managing rootworms, including soil-applied insecticides, foliar-applied insecticides, insecticide-treated seed, crop rotation and other transgenic options. However, there are resistance concerns with many of these tools. Corn rootworms have evolved resistance to broadcast soil insecticides (cyclodienes), aerial insecticides (methyl parathion) and crop rotation (through extended diapause and altered oviposition behavior). Some believe that the existing commercial transgenic option, expressing the Cry3Bb *Bt* protein, is at high risk for resistance evolution. The availability of novel corn rootworm protection tools lessens the selection pressure for pest adaptation to existing rootworm control methods. There is no significant sequence homology of either Cry34Ab1 or Cry35Ab1 with other registered PIPs (Cry1Ab, Cry1Ac, Cry1F), including the Cry3 family which is exploited in the only commercialized PIP (Cry3Bb) targeting corn rootworms. Cry34/35 is not expected to exhibit cross-resistance to any other currently available rootworm-control technology. By providing an additional option to corn growers for managing rootworms, Cry34/35Ab1 rootworm-protected corn will add to the sustainability of all tools.

VI.G. POTENTIAL CHANGES IN AGRICULTURAL PRACTICES ASSOCIATED WITH THE USE OF HERBICIDE TOLERANT CORN LINES

Approximately 80 million acres of corn are planted annually in the United States, and nearly all of these acres are treated with herbicide. Products are applied pre-plant, pre-emergence and post-emergence. In general, corn receives a soil applied herbicide application followed by a post-emergence application. Corn line 59122 is glufosinate-ammonium tolerant, and as such, provides an alternative weed management tool to growers. Glufosinate-ammonium is a broad spectrum, post-emergence herbicide. Such an herbicide could provide growers the opportunity to move away from pre-emergence, residually active compounds and could increase the amount of conservation and no-till acres of corn planted in the United States.

Corn line 59122 is still susceptible to other herbicides normally used to control corn should it appear as a volunteer weed in other crops. For example, in soybean, the crops most commonly rotated with corn, herbicides based on sulfonylurea, lipid biosynthesis inhibitors or Fluazifop/fomesafen could be used to control corn volunteers.

It is estimated that approximately 10 million acres of herbicide tolerant corn will be planted in 2000-2001. This includes other glufosinate- and glyphosate-tolerant corn

developed through recombinant technology as well as imidizolinone tolerant corn developed through selected mutagenesis and traditional plant breeding. If line 59122 crosses with corn lines expressing resistance to herbicides with different modes of action, corn volunteers with multiple herbicide resistance may emerge. However, the competition from the pollen load within a given field would keep the incidence of this very low. Additionally, various agronomic practices including appropriate variety selections and crop rotation, and rotation of herbicides with different modes of action can be used to avoid or manage volunteer corn resistant to one or a few herbicides.

Corn lines that contain both *B.t.* and herbicide tolerance have been on the market since 1997 and have had a positive impact on agricultural practices. These products have provided simple, inexpensive yet highly effective means of controlling lepidopteran pests; have been environmentally benign so they preserve beneficial insects, decrease cultivation needs, and require less total pounds of active pesticidal compounds (both insecticidal and herbicidal). Corn line 59122 is coleopteran specific and herbicide tolerant, therefore, it is anticipated that it will also provide the same benefits as the lepidopteran *B.t.* plants.

VI.H. VERTICAL TRANSFER OF THE INTRODUCED GENETIC MATERIAL

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

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

VI.I. HORIZONTAL TRANSFER OF THE INTRODUCED GENETIC MATERIAL

There is no known mechanism for, or definitive demonstration of, DNA transfer from plants to microbes (Nap *et al.*, 1992; Redenbaugh *et al.*, 1994). Even if such a transfer were to take place, transfer of *cry*34/35Ab1 or *pat* from line 15344 would not present a human health or plant pest risk. Genes encoding the PAT enzyme and similar acetyl transferases are found in nature. Similarly, *B.t.* Cry34/35Ab1 ICP was isolated from *Bacillus*

thuringiensis, a ubiquitous soil bacterium and therefore a variety of *cry* genes from *B.t.* can be found in nature. Cry34/35 itself has been shown to occur in geographically diverse settings associated with agricultural and non-agricultural lands. Recipients would, therefore, not pose a greater plant pest risk than the environmentally prevalent wild type microbes from which the genes originated.

The *spc* and *tet* genes coding for resistance to the antibiotics spectinomycin and tetracycline, respectively, were demonstrated to not be present in *B.t.* Cry34/35Ab1 corn line 59122. The T-DNA fragment (designated insert PHP17662) used to transform *B.t.* Cry34/35Ab1 corn line 59122 did not contain the *spc* or *tet* genes, although these genes were present on the original plasmid vector PHP17662. Therefore, there are no concerns for occurrence and effects of antibiotic resistance markers in the environment.

VII. ADVERSE CONSEQUENCES OF INTRODUCTION

The evidence provided in this petition supports the conclusion that *B.t.* Cry34/35Ab1 corn line 59122 presents low risk to human health and the environment and does not present a plant pest risk. In 2003, EPA issued a final rule granting Cry34Ab1 and Cry35Ab1 proteins a temporary exemption from the requirement of a tolerance in field corn when used as plant incorporated protectant (PIP) [Federal Register: July 7, 2003 (Volume 68, Number 129)][Rules and Regulations][Page 40178-40183]]. In temporarily exempting the Cry34Ab1 and Cry35Ab1 proteins, EPA concluded that "there is sufficient data available, considering all information on the Cry34Ab1 protein, to make a finding that there is a reasonable certainty that no harm will result from the aggregate exposure to the Cry34Ab1 and Cry35 Ab1 proteins as expressed in corn".

Data that were submitted demonstrated the lack of mammalian toxicity at high levels of exposure to the pure Cry34Ab1 and Cry35Ab1 proteins, alone and in mixtures. These data demonstrated the safety of the product at levels well above the maximum exposure levels that are reasonably anticipated in crops. Further, amino acid sequence comparisons submitted to EPA show no similarity of Cry34/35Ab1 to known allergenic proteins in public protein databases. Data were also submitted demonstrating that the Cry34Ab1 and Cry35Ab1 proteins were rapidly degraded by gastric fluid *in vitro* and that the proteins were not glycosylated. The proteins were also shown to be heat labile.

Based on exposure estimates and the results of the toxicological studies, there is low risk to non-target organisms and beneficial insects from expression of the Cry34/35Ab1 ICP in corn line 59122. *B.t.* Cry34/35Ab1 corn line 59122 exhibits typical agronomics and normal Mendelian inheritance of the introduced genetic material. There has been no evidence of increased susceptibility to insect pests or disease in corn 59122 when compared to conventional corn hybrids.

The PAT protein present in corn line 59122 is the expression product of the *pat* gene. The PAT protein has been studied extensively and has been found to be safe for consumption in food or feed (OECD, 1999). In the US, the EPA has established an

exemption from the requirement of a tolerance for residues of the plant-pesticide ingredients phosphinothicin acetyltransferase (PAT) and the genetic material necessary for its production in all plants (USEPA, 1997).

Hybrids derived from *B.t.* Cry34/35Ab1 corn line 59122 will be the first corn hybrids to use an ICP derived from *Bacillus thuringiensis* strain PS149B1. These hybrids demonstrate a spectrum of activity to the major corn rootworm pests including western corn rootworm, northern corn rootworm and Mexican corn rootworm.

IX. REFERENCES

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

Aldrich, S.R., Scott, W.O. and Hoeft, R.G. 1986. Modern Corn Production, 3rd Edition. A&L Publications.

An, G., Mitra, A., Choi, H.K., Costa, M.A., An, K., Thornburg, R.W., and Ryan, C. 1989. Functional analysis of the 3' control region of the potato wound-inducible proteinase inhibitor II gene. Plant Cell 1(1): 115-122.

Armstrong, C.L., Green, C.E. and Phillips, R.L. 1991. Development and Availability of Germplasm with High Type II Culture Formation Response. Maize Genetics Co-operation NewsLetter 65: 92-93.

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

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

Bauman, L.F. and Crane, P.L. 1985. The Corn Crop. NCH-29. Purdue University Cooperative Extension Service, West Lafayetter, IN.

Brussock, S.M. and Currier, T.C. 1990. Use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis to quantify *Bacillus thuringiensis*-endotoxins. Pages 78-87. In: American Chemical Society Symposium Series No. 432.

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

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

Codex Alimentarius Commission. 2001. Information paper on familiarity. Codex Ad Hoc Intergovernmental Task Force on Foods Derived from Biotechnology. CX/FBT/01/7, February, 2001. Joint FAO/WHO Food Standard Programme, Rome.

Crickmore, N.; Ziegler, D. R.; Feitelson, J.; Schnepf, E.; van Rie, J.; Lereclus, D.; Baum, J.; Dean, D. H. 1998. Revision of the nomenclature for the Bacillus thuringiensis pesticidal crystal proteins. Microbiol. Mol. Biol. Rev. 62:807-813.

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

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

Galinat, W.C. 1988. The Origin of Corn. pp. 1-31. In Sprague, G.F., Dudley, J. W., Editors. Corn and Corn Improvement, Third Edition. American Society of Agronomy, Crop Science Society of America, and Soil Science Society of America, Madison, Wisconsin. 986p.

Gao, Y. 2000. FA&PC 003143, Dow AgroSciences unpublished internal report.

Gendel, S. 1998. Sequence databases for assessing the potential allergenicity of proteins used in transgenic foods. Advances in Food and Nutrition Research 42: 63-92.

Glatt, C.M. 1999. Phosphinothricin acetyltransferase (PAT) protein: *In vitro* digestibility study. DuPont unpublished internal report. Study ID: DuPont-3365.

Guthrie, W.D., F.F. Dicke, and C.R. Neiswander. 1960. Leaf and sheath feeding resistance to the European corn borer in eight inbred lines of dent corn. *Ohio Agric. Res. Dev. Cent. Res. Bull.* 860.

Harris, D.L. 1969. An evaluation of pollen from corn and cotton as a diet for *Coleomegilla maculata* (DeGeer). M.S. thesis, University of Arkansas, Fayetteville.

Herman, R.A., Schafer, B.W., Korjagin, V.A., and Ernest, A.D. 2003. Rapid digestion of Cry34Ab1 and Cry35Ab1 in simulated gastric fluid. J. Agricultural and Food Chemistry 51: 6823-6827.

Herman, R.A., Scherer, P.N., Young, D.L., Mihaliak, C.A., Meade, T., Woodsworth, A.T., Stockhoff, B.A. and Narva, K.E. 2002. Binary insecticidal crystal protein from *Bacillus thuringiensis,* strain PS149B1: Effects of individual protein components and mixtures in laboratory bioassays. J. Econ. Entomol. 95: 635-639.

Herman, R.A., Scherer, P. N. and Wolt, J. D. 2002, Rapid degradation of a binary, PS149B1, delta-endotoxin of *Bacillus thuringiensis* in soil, and a novel mathematical model for fitting curve-linear decay. Environ. Entomol. 31: 208-214.

Hertig, C., Rebmann, G., Bull, J., Mauch, F. and Dudler, R. 1991. Sequence and tissue specific expression of a putative peroxidase gene from wheat (*Triticum aestivum* L.). Plant Mol. Biol. 16: 171-174.

Hohn, T., Richards, K., Genevieve-Lebeurier. 1982. Cauliflower mosaic virus on its way to becoming a useful plant vector. Current Topics in Microbiology and Immunology 96: 194-236.

Hitchcock, A.S. 1971. *Tripsacum* L. Gamagrass. In: Manual of the Grasses of the United States, pp. 790-792. Miscellaneous Publication 200, U.S. Department of Agriculture, 2nd Ed.

Hoffman, M.P. and Grodsham, A.C. 1993. Natural enemies of vegetable insect pests. Cooperative Extension, Cornell University, Ithaca, NY. (www.nysaes.cornell.edu/ent/biocontrol/predators/coloemeg.html)

Holt, J. G.; Krieg, N. R.; Sneath, P. H. A.; Bergey, D. H. 1993. Bergey's Manual of Determinative Bacteriology. 9th ed. Williams and Williams, Baltimore.

ILSI (International Life Sciences Institute). 2003. ILSI Crop Composition Database. www.cropcomposition.org. Accessed 8/12/2003.

Jugenhemier, R.W. 1976. Corn. Improvement, seed production, and uses. John Wiley and Sons, New York/London/Sydney/Toronto.

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

Langer, M., Rothe, M., Eck, J., Mockel, B. and Zinke, H. 1996. A Non-radioactive Assay for Ribosome-Inactivating Proteins. *Analyt. Biochem*. 243:150-153.

Li, X.; Chang, Y.H. 1995. Amino-terminal protein processing in *Saccharomyces cerevisiae* is an essential function that requires two distinct methionine aminopeptidases. Proc. Natl. Acad. Sci. U.S.A. 19;92(26):12357-61.

Martin, P. A. W.; Travers, R. S. 1989. Worldwide abundance and distribution of Bacillus thuringiensis isolates. Applied Environ. Microbiol. 55, 2437-2442.

Mendelsohn, M., Kough, J., Zigfridas, V., Matthews, K. 2003. Are Bt crops safe? Nature Biotech. 21: 1003-1009.

Meyer, T. 1999. Comparison of amino acid sequence similarity of Cry1F and PAT proteins to known allergen proteins. Unpublished report of Pioneer Hi-Bred International. Study ID: PHI99-013. 24p.

Monsanto Company. 2001. Safety assessment of YieldGard® insect-protected corn event MON 810. www.monsantoinfo.dk/nyhedsbrev/YieldGardCornProductSafetySummary.pdf.

Murray, E., Lotzer, J., and Eberle, M. 1989. Codon usage in plant genes. Nucleic Acid Research 17(2): 477-498.

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

OECD. 2002. Consensus document on compositional considerations for new varieties of maize (*Zea mays*): Key food and feed nutrients, anti-nutrients and secondary plant metabolites. ENV/JM/MONO(2002)25. 42p.

OECD (Organization for Economic Cooperation and Development). 1993. Safety Considerations for Biotechnology: Scale-up of Crop Plants. Paris: OECD.

Ohio Corn Marketing Program. 2000. The Many Uses of Corn. At: www.ohiocorn.org/usage/uses.htm.

Oleson, J. D.1998. Joint Meeting, Entomological Society of America and American Phytopathological Society, Display Presentation, D428 Linear Scale for Evaluating Corn Rootworm Larval Injury. November 8-12. Las Vegas NV.

Pilcher, C.D. 1997. Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. Environmental Entomology, Vol. 26, no. 2: 446-454.

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

Rawn, J.D. 1989. The Michaelis-Menton rate law governs enzymatic reactions. Pp. 167-173. In: Biochemistry. Neil Patterson Publishers, Burlington, NC.

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

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

Schmidt, J. 2003. Certificate of Analysis for Cry34Ab1 protein standard lot # 100501-F80. Unpublished report of Pioneer Hi-Bred International.

Shanahan, D. and Stauffer, C. 2000. Petition for determination of non-regulated status *B.t.* Cry1F insect-resistant, glufosinate-tolerant maize line 1507 (00-136-01p). 67p.

Sims, S.R., and Holden, L.R. 1996. Insect bioassay for determining soil degradation of *Bacillus thuringiensis* subsp. *kurstaki* Cry1Ab protein in corn tissue. Phssio.Chem.Ecol. Vol. 25(3): 659-664.

Smith, B.C. 1961. Results of rearing some coccinellid (Coleoptera: Coccinellidae) larvae on various pollens. Proc. Entomol. Soc. Ont. 91: 270-271.

Smith, S.F. and Krischik, V.A. 1999. Effects of systemic imidacloprid on *Coleomegilla maculata* (Coleoptera: Coccinellidae). Environmental Entomology, Vol. 28: 1189-1195.

Song, P. 2003. Comparison of the amino acid sequences of *Bacillus thuringiensis* strain PS149B1 Cry34Ab1 and Cry35Ab1 insecticidal crystal proteins as expressed in maize to known protein allergens. Study ID GH-C 5671. Dow AgroSciences Unpublished Internal Report.

United States Department of Agriculture. 1995. Availability of Determination of Nonregulated Status for Genetically Engineered Corn. Animal and Plant Health Inspection Service. Volume 60, Number 134, pp. 36095-36096.

United States Department of Agriculture, Economic Research Service. 2000. Feed Outlook. January 14, 2000.

United States Department of Agriculture – NASS Agricultural Statistics. 2000. U.S. Government Printing Office, ISBN 0-16-036158-3. pp. I-24 to I-31.

United States Fish and Wildlife Service. 1991. American burying beetle (*Nicrophorus americanus*) recovery plan. Newton Corner, Massachusetts. 80p. http://ecos.fws.gov/recovery_plan/pdf_files/1991/910927.pdf

USEPA (United States Environmental Protection Agency). 2001. Reregistration Eligibility Decision (RED): *Bacillus thuringiensis (Bt)* Plant-Incorporated Protectants. Office of Pesticide Programs, Biopesticides and Pollution Prevention Division, Washington, DC. October 15, 2001. p. IIB2.

USEPA (United States Environmental Protection Agency). 1998. Reregistration Eligibility Decision (RED): Bacillus thuringiensis. Office of Prevention, Pesticides, and Toxic Substances, Washington, DC. EPA738-R-98-004, March, 1998.

USEPA. 1997. Terms of Environment. EPA175B97001. USEPA, OC, Washington, DC. Accessed electronically 21 Oct 2002. http://www.epa.gov/OCEPAterms/

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

Watson, S. A. 1982. Corn: Amazing maize. General Properties. pp 3-29 in CRC Handbook of Processing and Utilization in Agriculture, vol II, Part 1 Plant Products I. A. Wolf (ed.) CRC Press Inc., Florida

Watson, S. A. 1987. Structure and Composition. pp 53-82 in Corn: Chemistry and Technology. S. A. Watson and P. E. Ransted (eds.) American Association of Cereal Chemists, Inc., Minnesota

Weber, N. and Igo, E. 2003. Characterization of transgenic corn event DAS-59122-7 to investigate the genetic equivalence of the inserted DNA within a single generation. Pioneer Hi-Bred International, Inc. Unpublished internal report. 43p.

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

Zupan, J.R., and Zambryski, P. 1995. Transfer of T-DNA from *Agrobacterium* to the plant cell. Plant Physiol. 107: 1041-1047.

Zupan, J. , and Zambryski, P. 1997. The *Agrobacterium* DNA transfer complex. Crit. Rev. Plant Science 16: 279-295.

APPENDIX 1. CRY34/35AB1: DIETARY RISK ASSESSMENT

PIONEER
A DUPONT COMPANY

DuPont Agriculture & Nutrition
Pioneer Emerson Building
7250 N.W. 62nd Ave.
P.O. Box 552
Johnston, IA 50131-0552
(515) 270 3202 Tel

Memo

From: Raymond Layton
To: Larry Zeph
Date: 6 November 2003

Cry34/35 Maize (DAS-59122-7): Dietary Risk Assessment

Background:

Maize (*Zea mays*) has been genetically modified through the insertion of two genes (*cry*34Ab1 and *cry*35Ab1) from *Bacillus thuringiensis* (PS149B1), which encode for the two proteins (Cry34/35Ab1). Together these proteins act to protect the plant from western corn rootworm (*Diabrotica virgifera virgifera*), a major pest of maize.

The apparent site of action of Cry34/35Ab1 is the insect midgut where the proteins disrupt epithelial cells. This site of action is consistent with that of other Cry toxins. The precise mode of action has not been determined; however, laboratory studies have shown that the mode of action is not related to protein synthesis. The proteins are related to insecticidal proteins encoded by *B. thuringiensis* and *B. sphaericus* (Cressman 2003). Cry35Ab1 has predicted structural homology or similarity with other proteins containing the $(QxW)_3$ carbohydrate-binding domain (Pfam 2003). Proteins with the $(QxW)_3$ domain are members of a large "family" of proteins that have diverse functions (this particular protein family is named the "ricin b lectin family" after one of its members). Cry34Ab1 has structural homology to equinatoxin II, a pore forming toxin found in sea anemones (unpublished data). It is not unexpected that these proteins share binding domains and structural homology with other proteins that have similar functions. The Cry34/35Ab1 proteins have a narrow spectrum of activity among insects and other organisms that have been tested; the spectrum of activity appears to be restricted to coleopteran larvae.

Toxicity and Allergenicity Studies:

Toxicity data are available from tests conducted using mice and broiler chickens. Mice were dosed using a single dose gavage followed by a 14-day observation period. Microbially-derived Cry34Ab1 (2700 mg/kg bw) and Cry35Ab1 (1850 mg/kg bw) were administered separately and in combination (482 mg/kg bw and 1520 mg/kg bw for Cry34Ab1 and Cry35Ab1, respectively). Parameters evaluated included body weights and detailed clinical evaluations. No treatment related effects were seen during any of the studies (Brooks and DeWildt 2000a, 2000b, 2000c).

A chicken broiler study was conducted using grain from event DAS-15344. Two diets, starter and grower, were formulated containing approximately 60% maize. The birds were observed three times each day for overall health, behavioral changes and/or evidence of toxicity. Body weights and feed weights were measured every 7 days. The body weight parameters evaluated at the end of the 42-day study included carcass yield, thighs, breasts, wings, legs, abdominal fat, kidneys, and whole liver. No treatment related effects were seen in this study (Smith 2003).

Several studies were conducted to evaluate the potential for Cry34/35Ab1 to have allergenic effects. Amino acid sequences of Cry34/35Ab1 were compared to amino acid sequences of putative or known allergens. No matches were found to eight or more contiguous amino acids and CGA FASTA comparisons revealed no matches of 35% or greater over 80 amino acid residues (Song 2003). Cry34/35Ab1 have been shown to be heat labile, indicating that these proteins will lose activity under most conditions found during processing and cooking of the grain (Shan and Schafer 2003). Simulated gastric digestion studies indicated that both Cry34/35Ab1 are readily digested (Cry34 - 90% digestion in less than 7 minutes, Cry35 digested

within 5 minutes) under simulated gut conditions (Korjagin and Ernest 2000, Korjagin et al. 2002, Herman et al. 2002). The results of the amino acid similarity, heat lability, and digestion studies indicated that Cry34Ab1 or Cry35Ab1 are unlikely to be human allergens.

Exposure Characterization:

Cry34Ab1 and Cry35Ab1 are expressed in plant tissues, including grain (Essner 2003). Since maize grain is a highly mixed and processed commodity, average expression values (combined data from sprayed and unsprayed maize) can be used in conservatively characterizing exposure:

Average expression of proteins in grain (ng/mg dry weight*)

Event	Cry34Ab1	Cry35Ab1
DAS-59122-7	55.39	0.95

(*Concentrations in maize would be slightly lower due to higher moisture content in typical maize.)

The actual exposure to Cry34/35Ab1 proteins in the diet is expected to be much lower than these values because: (1) Protein degradation is likely to occur during transport and storage. (2) Grain containing Cry34/35Ab1 will be mixed with other grain not containing these proteins. (3) Reductions in protein concentrations will occur during processing to produce high fructose corn syrup and vegetable oils (commodities that contain negligible levels of protein).

Risk Characterization:

A simple way to characterize dietary risk is to calculate the amount of a food that would have to be eaten to expose a person to the same level of protein that was used in the toxicology studies. The estimated amount of food is then evaluated in terms of how feasible it would be to eat that amount of food in one day.

For example, Cry34Ab1 expression in DAS-59122-7 grain is approximately 55 ng/mg dry weight. This means that one milligram of grain contains 0.000055 mg of protein. Mice were dosed using 2700 mg of microbially produced protein per kilogram body weight. To receive an equivalent dose of Cry34Ab1 protein in grain, each mouse would have had to eat 48.7 kg of grain per kg body weight. Similar estimates can be made as well for Cry35Ab1. Following these assumptions, one can calculate the amount of raw grain that would have to be eaten by an infant, a child, or an adult to match the proteins levels used in the mouse studies.

	Cry34Ab1 Alone	Cry35Ab1 Alone	Cry34Ab1 Combination	Cry35Ab1 Combination
Expression (ng/mg)	55.39	0.95	55.39	0.95
Tox Test Dose (mg/kg bw)	2700	1850	482	1520
Amount (kg/day) of grain needed to be consumed to match dose in toxicology tests:				
Child (10 kg)	487	19474	44	8000
Adult (60 kg)	2925	116842	522	96000

Based on these simplistic worst-case calculations, it is obvious that there is a clear margin of safety for these proteins. The actual margin of safety will be much greater when the effects of factors such as market share and processing are taken into account.

In addition to the worst case risk assessment for the protein studies, a comparison can also be made between the amount of maize consumed in the poultry study and known maize consumption by humans for various populations around the world. The poultry study serves as an acceptable indicator of potential effects because the birds have a very high maize consumption level (approximately 60%) and are growing very rapidly. Maize consumption data for various subpopulations was taken from the GEMS/FAO (2003) dietary tables.

	Middle Eastern	Far Eastern	African	Latin American	European
Consumption					
Maize Flour (g/person/d)	31.8	31.2	106.2	40.3	8.8
Total Diet (g/person/day)	1346.1	1093.8	1045.3	1354.1	1907.6
Assuming 60kg bw					
Maize Flour (g/person/d)	0.5	0.5	1.8	0.7	0.1
Total Diet (g/kg bw/day)	22.4	18.2	17.4	22.6	31.8
Percent of total diet					
Maize Flour	2.4%	2.9%	10.2%	3.0%	0.5%

Except in Africa, maize consumption makes up three percent or less of the total diet on a grams per day basis. Even in Africa, maize consumption represents only 10% of the total dietary consumption. This much lower than the proportion of maize in the diets used in the poultry study (approximately 60%). When the market share and processing effects on the proteins are taken into account, the exposures in the poultry study are probably several orders of magnitude higher than expected exposures in human populations.

Conclusions:

- The site of action (insect midgut epithelial cells) of Cry34/35Ab1 appears to be similar to other insecticidal proteins. Cry34/35Ab1 have a narrow spectrum of activity.
- Based on the lack of effects seen in the toxicology tests, Cry34/35Ab1 appear to be essentially non-toxic to mammals and poultry.
- Large margins of safety exist for these proteins, even when using simplistic, worst-case assumptions.

References Cited

Bateman, A., E. Birney, L. Cerruti, R. Durbin, L. Etwiller, S.R. Eddy, S. Griffiths-Jones, K.L. Howe, M. Marshall, and E.L.L. Sonnhammer. 2002. The Pfam protein families database. Nucleic Acids Research 30(1):276-280.

Brooks, J.J. and P.M. DeWildt. 2000a. PS149B1 14 Kd and 44 Kd polypeptides: acute oral toxicity study in CD-1 mice. Unpublished Dow Chemical Company report, study number 001128.

Brooks, J.J. and P.M. DeWildt. 2000b. PS149B1 14 Kd polypeptide: acute oral toxicity study in CD-1 mice. Unpublished Dow Chemical Company report, study number 001129.

Brooks, J.J. and P.M. DeWildt. 2000c. PS149B1 44 Kd Polypeptide: acute oral toxicity study in CD-1 mice. Unpublished Dow Chemical Company report, study number 001130.

Cressman, R.F. 2003. Evaluation of the Sequence Similarities of the Cry34Ab1, Cry35Ab1, and PAT proteins to the public protein sequence databases. Unpublished DuPont/Pioneer Hi-Bred International report, study number PHI-2003-046.

Essner, R. 2003. Agronomic characteristics, quantitative ELISA and nutrient composition analysis of hybrid maize lines containing the *cry24Ab1, cry35Ab1,* and *pat* genes: Chile locations. Unpublished Pioneer Hi-Bred International report, study number PHI-2002-050.

GEMS/FAO. 2003. http://www.who.int/foodsafety/publications/chem/en/regionaldiets_table.pdf.

Herman, R.A., B.W. Schafer, and V.A. Korjagin. 2002. SDS-PAGE sensitivity analysis for Cry35Ab1 in support of the simulated gastric fluid digestion study MRID#45242212. Unpublished Dow AgroSciences report, study number GH-C 5513.

Korjagin, V.A. and A.D. Ernest. 2000. *In vitro* digestibility of PS149B1 proteins. Unpublished Dow AgroSciences report, study number 000302.

Korjagin, V.A., R.A. Herman, P.L. Hunst, and B.W. Schafer. 2002. *In vitro* simulated gastric fluid digestibility study of microbially derived Cry34Ab1 protein. Unpublished Dow AgroSciences report, study number 01011.

Pfam. 2003. Pfam 10.0 (July 2003, 6190 families). Pfam is a large collection of multiple sequence alignments and hidden Markov models covering many common protein families. Pfam version 10.0 (July 2003) contains alignments and models for 6190 protein families, based on the Swissprot 41.10 and SP-TrEMBL 23.15 protein sequence databases. Accessed at http://pfam.wustl.edu/

Shan, G. and B.W. Schafer. 2003. Heat lability of insecticidal crystal proteins Cry34Ab1 and Cry35Ab1. Unpublished Dow AgroSciences report, study number 030088.

Smith, B. 2003. Nutritional Equivalency Study of PS149B1 Maize: Poultry Feeding Study. PHI-2001-043

Song, P. 2003. Comparison of the amino acid sequences of *Bacillus thuringiensis* strain PS149B1 Cry34 and Cry35 (synpro) insecticidal crystal proteins as expressed in maize to known protein allergens. Unpublished Dow AgroSciences report, study number GH-C 5671.

APPENDIX 2. STUDY REPORTS SUPPORTING THE REGULATORY APPROVAL OF *B.T.* CRY34/35AB1 MAIZE LINE 59122.

CBI-Deleted COPY

All study reports contained in this appendix are <u>Confidential Business Information</u>.

Appendix 2 – Section 1

[CBI-Deleted]

Narva, K., Schnepf, H.E. and Wolt, J.D. 2003. Cry34/35 Protein Distribution and Familiarity. Study ID: GH-C 5702. Dow AgroSciences LLC unpublished internal report. 26p.

Appendix 2 – Section 2

[CBI-Deleted]

Essner, R. 2003. Agronomic Characteristics, Quantitative ELISA and Nutrient Composition Analysis of Hybrid Maize Lines Containing *cry*34Ab1, *cry*35Ab1 and *pat* genes. Study ID: PHI-2002-050. Pioneer Hi-Bred unpublished internal report. 1971p.

Appendix 2 – Section 3

[CBI-Deleted]

Herman, R.A. and Babcock, J.M. 2003. Biological Equivalency of Cry34/35Ab1 Insecticidal Crystal Protein in Transgenic Plants Derived from Transgenic *Pseudomonas fluorescens.* Study ID: GH-C 5696. Dow AgroSciences unpublished internal report. 17p.

Appendix 2 – Section 4

[CBI-Deleted]

Schafer, B.W., Collins, R.A., Schwedler, D.A. and Xu, X.U. 2003. Characterization of Cry34Ab1 and Cry35Ab1 proteins from transgenic maize event E4497.59.1.22 (DAS-59122-7). Study ID: 030033. Dow AgroSciences unpublished internal report. 50p.

Appendix 2 – Section 5

[CBI-Deleted]

Schafer, B.W. and Collins, R.A. 2003. Characterization of Phosphinothricin Acetyltransferase (PAT) Derived from Transgenic Maize Event E4497.59.1.22. Study ID: 030027. Dow AgroSciences unpublished internal report. 30p.

Appendix 2 – Section 6

[CBI-Deleted]

Locke, M.E. and Igo, E. 2003. Characterization of DNA Inserted into Transgenic Corn Events DAS-45216-6 and DAS-59122-7. Study ID: PHI-2002-038. Pioneer Hi-Bred unpublished internal report. 81p.

Appendix 2 – Section 7

[CBI-Deleted]

Locke, M.E., Weber, N. and Dietrich, N. 2003. Detailed Characterization of DNA Inserted into Transgenic Corn Events DAS-45216-6 and DAS-59122-7. Study ID: PHI-2002-041. Pioneer Hi-Bred unpublished internal report. 62p.

Appendix 2 – Section 8
[CBI-Deleted]

Schafer, B.W., Yau, K. and Herman, R.A. 2003. Evaluation of Microbe-Derived Cry34Ab1 and Cry35Ab1 Proteins for Protein Synthesis Inhibition Activity. Study ID: 031139. Dow AgroSciences unpublished internal report. 37p.

Appendix 2 – Section 9

[CBI-Deleted]

Smith, B. 2003. Nutritional Equivalency Study of Maize Containing Cry34Ab1 and Cry35Ab1: Poultry Feeding Study. Study ID: PHI-2001-043. Pioneer Hi-Bred unpublished internal report. 33p.

Appendix 2 – Section 10

[CBI-Deleted]

Higgins, L. 2003. The Effect of Cry34Ab1/Cry35Ab1 Proteins on the Development and Mortality of the Ladybird Beetle, *Coleomegilla maculate* De Geer. Study ID: PHI-2003-045. Pioneer Hi-Bred unpublished internal report. 97p.

Appendix 2 – Section 11

[CBI-Deleted]

Poletika, N.N. 2003. Non-target Invertebrate Ecological Risk Assessment for Field Corn Expressing Cry34Ab1 and Cry35Ab1 Insecticidal Crystal Protein in Event DAS-59122-7. Study ID: GH-C 5681. Dow AgroSciences unpublished internal report. 98p.

Appendix 2 – Section 12

[CBI-Deleted]

Higgins, L. 2003. Evaluation of the Impact of Corn Rootworm Control Strategies on Non-target Arthropods. Study ID: PHI-2001-020. Pioneer Hi-Bred unpublished internal report. 85p.

Appendix 2 – Section 13

[CBI-Deleted]

Storer, N.P., Lefko, S.A., Babcock, J.M., Edwards, J.M. and Binning, R.R. 2003. Investigations into Dose of Cry34Ab1/Cry35Ab1 Rootworm-Resistant Maize Event DAS-59122-7 Against Western and Norther Corn Rootworms in Support of Trait Durability Plan. Study ID: GH-C 5687. Dow AgroSciences unpublished internal report. 60p.

Appendix 2 – Section 14

[CBI-Deleted]

Storer, N.P. and Lang, B.A. 2003. Effect on Western Corn Rootworm Adults of Feeding on Cry34/35Ab1 Corn Rootworm-Protected Corn Tissue and Implications for Product Durability. Study ID: GH-C 5686. Dow AgroSciences unpublished internal report. 45p.

Appendix 2 – Section 15

[CBI-Deleted]

Higgins, L. 2003. Evaluation of Endangered/Threatened Insect Species Relative to the Use of Cry34Ab1/Cry35Ab1 Corn Rootworm-Resistant Maize Hybrids. Study ID: PHI-2003-025. Pioneer Hi-Bred unpublished internal report. 407p.

Appendix 2 – Section 16

[CBI-Deleted]

Storer, N.P. and Lefko, S.A. 2003. Trait Durability Plan for Cry34/35-Corn Rootworm-Protected Corn Event DAS-59122-7 Following Commercialization. Study ID: GH-C 5689. Dow AgroSciences unpublished internal report. 161p.

Appendix 2 – Section 17

[CBI-Deleted]

Herman, R.A. 2000. Microbial PS149B1 Binary Delta-Endotoxin: Maize-Insect-Pest Susceptibility Study. Study ID: 000366 (GH-C 5114). Dow AgroSciences unpublished internal report. 29p.

Appendix 2 – Section 18

[CBI-Deleted]

Higgins, L. 2000. The Tri-Trophic Interaction Between PS149B1 Transformed Maize, Corn Leaf Aphid and Ladybird Beetle. Study ID: PHI-2000-022. Pioneer Hi-Bred unpublished internal report. 145p.

Appendix 2 – Section 19

[CBI-Deleted]

Korjagin, V.A. and Ernest, A.D. 2000. *In Vitro* Digestibility of PS149B1 Proteins. Study ID: 000302 (GH-C 5132). Dow AgroSciences unpublished internal report. 37p.

Appendix 2 – Section 20

[CBI-Deleted]

Bryan, R.L. *et al.*, 2000. PS149B1 Binary Insecticidal Crystal Protein: A Dietary Toxicity Study with the Ladybird Beetle. Study ID: 379-103 (000155). Dow AgroSciences unpublished internal report. 18p.

Appendix 2 – Section 21

[CBI-Deleted]

Brooks, K.J. and De Widt, P.M. 2000. PS149B1 14 KDA Protein: Acute Oral Toxicity Study in CD-1 Mice. Study ID: 001130. The Dow Chemical Company unpublished internal report. 39p.

Appendix 2 – Section 22

[CBI-Deleted]

Brooks, K.J. and DeWidt, P.M. 2000. PS149B1 44 KDA Protein: Acute Oral Toxicity in CD-1 Mice. Study ID: 001129. The Dow Chemical Company unpublished internal report. 39p.

Appendix 2 – Section 23

[CBI-Deleted]

Brooks, K.J. and DeWidt, P.M. 2000. PS149B1 14 KDA and 44 KDA Proteins: Acute Oral Toxicity in CD-1 Mice. Study ID: 001128. The Dow Chemical Company unpublished internal report. 45p.

Appendix 2 – Section 24

[CBI-Deleted]

Stelman, S.J. 2000. Comparison of the Amino Acid Sequence of the *Bacillus thuringiensis* Strain PS149B1 13.6 kDa and 43.8 kDa Insecticidal Crystal Proteins to Known Protein Allergens. Study ID: GH-C 5140. Dow AgroSciences unpublished internal report. 188p.

Appendix 2 – Section 25

[CBI-Deleted]

Maggi, V. 2001. Microbial PS149B1 Binary Insecticidal Crystal Protein, Pollen Expressing PS149B1 Binary Insecticidal Crystal Protein and Individual PS149B1 14 kDa and 44 kDa Insecticidal Crystal Proteins: Evaluation of Dietary Exposure on Honeybee Development. Study ID: CAR 149-00. Dow AgroSciences unpublished internal report. 58p.

Appendix 2 –Section 26

[CBI-Deleted]

Herman, R.A. 2000. Thermolability of PS149B1 Binary Delta-Endotoxin. Study ID: 001041 (GH-C 5145). Dow AgroSciences unpublished internal report. 18p.

Appendix 2 – Section 27

[CBI-Deleted]

Bryan, R.L., Porch, J.R. and Krueger, H.O. 2000. PS149B1 Binary Insecticidal Crystal Protein: Acute Toxicity to the Earthworm in an Artifical Substrate. Study ID: 379-104. Dow AgroSciences unpublished internal report. 22p.

Appendix 2 – Section 28

[CBI-Deleted]

Herman, R.A. 2002. Heat Lability of Individual Proteins of the PS149B1 ICP. Study ID: GH-C 5360. Dow AgroSciences unpublished internal report. 15p.

Appendix 2 – Section 29

[CBI-Deleted]

Korjagin, V.A., Herman, R.A., Hunst, P.L. and Schafer, B.W. 2002. *In Vitro* Simulated Gastric Fluid Digestibility Study of Microbially-Derived Cry34Ab1 Protein. Study ID: GH-C 5361. Dow AgroSciences unpublished internal report. 95p.

Appendix 2 – Section 30

[CBI-Deleted]

Schafer, B.W. 2002. Characterization of Cry34Ab1 and Cry35Ab1 from Recombinant *Pseudomonas fluorescens* and Transgenic Maize. Study ID: GH-C 5545. Dow AgroSciences unpublished internal report. 17p.

Appendix 2 – Section 31

[CBI-Deleted]

Marino, T.A. and Yaroch, A.M. 2002. PS149B1 Binary Insecticidal Crystal Protein: An 8-Day Dietary Study with the Rainbow Trout, *Oncorhynchus mykiss*, Waldbaum. Study ID: 011193. The Dow Chemical Company unpublished internal report. 31p.

Appendix 2 – Section 32

[CBI-Deleted]

Marino, T.A. and Yaroch, A.M. 2001. PS149B1 Binary Insecticidal Crystal Protein: An Acute Toxicity Study with the Daphnid, *Daphnia magna* Straus. Study ID: 011137. Dow AgroSciencies unpublished internal report. 28p.

Appendix 2 – Section 33

[CBI-Deleted]

Porch, J.R. and Krueger, H.O. 2001. PS149B1 Binary Insecticidal Crystal Protein: Dietary Toxicity to Parasitic Hymenoptera (*Nasonia vitripennis*). Study ID: 011105 (379-115). The Dow Chemical Company unpublished internal report. 27p.

Appendix 2 – Section 34

[CBI-Deleted]

Teixeira, D. 2001. Assessment of Chronic Toxicity of Diet Containing *Bacillus thuringiensis* PS149B1 Insecticidal Crystal Protein to Collembola (*Folsomia candida*). Study ID: 011106. Dow AgroSciences unpublished internal report. 35p.

Appendix 2 – Section 35

[CBI-Deleted]

Sindermann, A.B., Porch, J.R. and Krueger, H.O. 2001. PS149B1 Insecticidal Crystal Protein: Dietary Toxicity to Green Lacewing Larvae (*Chrysoperla carnea*). Study ID: 379-116A. Dow AgroSciences unpublished internal report. 30p.

Appendix 2 – Section 36

[CBI-Deleted]

Herman, R.A., Schafer, B.W. and Korjagin, V.A. 2002. SDS-PAGE Sensitivity Analysis for Cry35Ab1 in Support of Simulated Gastric Fluid Digestion Study MRID#45242212. Study ID: GH-C 5513. Dow AgroSciences unpublished internal report. 11p.

Appendix 2 – Section 37

[CBI-Deleted]

Herman, R.A., Collins, R.A. and Young, D.L. 2000. Degradation of Microbial Binary PS149B1 Delta-Endotoxin in a Representative Soil from the Mid-Western USA Maize-Growing Region. Study ID: 000365 (GH-C 5113). Dow AgroSciences unpublished internal report. 26p.

Appendix 2 – Section 38

[CBI-Deleted]

Higgins, L. 2002. Field Efficacy of Cry34Ab1/Cry35Ab1 Maize Events Against Corn Rootworms. Study ID: PHI-2002-056. Pioneer Hi-Bred unpublished internal report. 12p.

Appendix 2 – Section 39

[CBI-Deleted]

Herman, R.A. and Hunst, P.L. 2002. Summary of Heat Lability Studies with Cry34Ab1/Cry35Ab1. Study ID: GH-C 5603. Dow AgroSciences unpublished internal report. 21p.

Appendix 2 – Section 40

[CBI-Deleted]

Korjagin, V.A. 2000. Characterization of *Pseudomonas*-Produced and Transgenic Maize Expressed Phosphinothricin. Study ID: 000369 (GH-C 5128). Dow AgroSciences unpublished internal report. 33p.

Appendix 2 – Section 41

[CBI-Deleted]

Gao, Y., Herman, R.A., Gilbert, J.R. and Patterson, V.L. 2000. Equivalency of Microbial- and Maize-Expressed PS149B1 Proteins. Study ID: 000367 (GH-C 5126). Dow AgroSciences unpublished internal report. 90p.

Attachment 1. USDA APHIS release notifications relevant to the field testing of *B.t.* Cry34/35Ab1 maize line 59122.

Dow AgroSciences LLC release notifications:

Internal ID	USDA #	County and State of Release
MS217a	01-277-06n	Maui, HI
MS244	02-060-21n	Maui, HI
MS245	02-060-22n	York, NE
MS246	02-060-23n	Story, IA; Columbia, WI; Benton, IN; Tipton, IN
MS255	02-162-08n	Maui, HI
MS273	03-008-06n	Maui, HI
279-CR-CRN-MR	03-035-15n	Cass, IA; Sac, IA; Scott, IA; Story, IA; Washington, IA; Wright, IA; DeKalb, IA; Macon, IL; McDonough, IL; Piatt, IL; Wayne, IL; Woodford, IL; Benton, IN; Marshall, IN; Sullivan, IN; Tipton, IN; Vanderburgh, IN; Butler, NE; Phelps, NE; York, NE; Miami, OH; Columbia, WI; Jefferson, WI
280-CR-CRN-MR	03-035-16n	Maui, HI
281-CR-CRN-R	03-052-09n	Freeborn, MN; Jackson, MN; Pipestone, MN; Redwood, MN

Pioneer Hi-Bred International, Inc. release notifications:

USDA #	Tracking #	Field Trial Location	Company
01-022-04R	CRN-US-2001-0002	Kekaha, HI	Pioneer
02-023-01R	CRN-US-2002-0001	Woodland, CA	Pioneer
		Rochelle, IL	
		Johnston, IA	
		Miami, MO	
		York, NE	
		Salinas, PR	
		Beeville, TX	
		Weslaco, TX	
02-023-02R	CRN-US-2002-0002	Kekaha, HI	Pioneer
03-022-01R	CRN-US-2003-0012	Princeton, IL	Pioneer
		Champaign, IL	
		Plainview, TX	
		Weslaco, TX	
		Marion, IA	
		Macomb, IL	
		Mitchell, IA	
		Union City, TN	
		Rochelle, IL	
		Johnston, IA	
		Janesville, WI	
		Salinas, PR	
		Miami, MO	
		Santa Isabel, PR	
		Tipton, IN	
		Woodland, CA	
		York, NE	
03-022-02R	CRN-US-2003-0013	Kekaha, HI	Pioneer