95-195-01p.

July 13, 1995

Dr. Arnold S. Foudin
Deputy Director
Biotechnology Permits
BBEP
USDA-APHIS
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Subject: Petition for Determination of Nonregulated Status For:
Insect Protected Corn (*Zea mays* L.) Expressing the Cry IA(b) Gene from *Bacillus thuringiensis* var. *kurstaki*

Dear Dr. Foudin:

Enclosed are two copies of the subject petition. One copy contains confidential business information and the other does not. Also enclosed is a justification for designating some of the information confidential.

The petition seeks nonregulated status for corn which expresses a modified form of the Cry IA(b) gene of *Bacillus thuringiensis* var. *kustaki* (Btk corn). Northrup King has tested inbreds and hybrids descended from the originally transformed corn which was crossed with elite Northrup King inbreds. Field testing of Btk corn has been conducted since 1992 under the permits or notifications listed on the enclosed and issued by USDA-APHIS. For the past two years, field trials have also been conducted under an EPA experimental use permit, number 67979-EUP-1.

Corn expressing the Btk protein has shown excellent resistance to European corn borer and is being evaluated for resistance to other lepidoptera. No plant pest characteristics have been observed.

Therefore, we are herewith requesting that insect protected corn (Zea *mays* L.) expressing the CryIA(b) gene from *Bacillus thuringiensis* var. *kurstaki* and

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descended from transformation event designated Bt11 be designated a nonregulated article under 7CFR 340.

If there are any questions concerning the enclosed or if additional information is needed, please do not hesitate to contact me.

Sincerely,

Diana G. Williams
Manager, Government Regulations

cc: E. Resler

Northrup King Co.'s
USDA-APHIS Permits/Notifications Covering the Release of
Insect Protected Corn (*Zea mays* L.)
Expressing the Cry IA(b) Gene from *Bacillus thuringiensis* var. *kurstaki.*

1992 - 1994

92-017-03
92-169-02

93-014-03
93-127-01N
93-237-03N
93-238-01N

94-069-01N
94-080-04N
94-080-05N
94-112-01N
94-237-01N
94-245-02N
94-347-04N

1995

95-052-05N
95-058-17N
95-068-04N
95-060-02N
95-060-03N
95-068-05N
95-065-13N
95-068-06N
95-065-14N
95-068-07N
95-068-08N
95-068-09N
95-065-15N
95-065-16N
95-068-10N
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NON-CONFIDENTIAL

PETITION FOR DETERMINATION OF NONREGULATED STATUS FOR:

Insect Protected Corn (*Zea mays* L.) Expressing the CryIA(b) Gene from *Bacillus thuringiensis* var. *kurstaki*

The undersigned submits this petition under 7 CFR 340.6 to request that the Director, BEEP, make a determination that this article should not be regulated under 7 CFR part 340.

Submitted by: Diana G. Williams
Manager, Government Regulations
Northrup King Co.
7500 Olson Memorial Highway
Golden Valley, MN 55427
612-593-7285

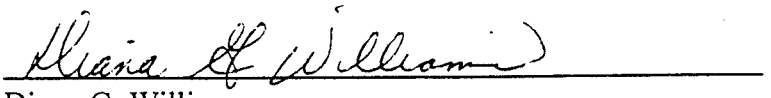
This date: 13 July 1995

Prepared by: William P. Pilacinski and Diana G. Williams

Contributors: B. J. Bolan, M. D. Edwards, R. A. Evans, D. Farrar, J. A. Hanten, D. Mead, I. J. Mettler, D. W. Mies, J. L. Rosichan, J. B. Sagers, and A. S. Wang

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.



Diana G. Williams
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STATEMENT OF GROUNDS

I. Introductory Discussion

The regulated article for which this petition is being made is insect protected corn (*Zea mays* L.) expressing the CryIA(b) gene from *Bacillus thuringiensis* var. *kurstaki*. The active ingredient produced within the corn plant is a truncated form of the delta-endotoxin protein of the soil microorganism, *Bacillus thuringiensis* var. *kurstaki* (Btk) strain HD-1. The gene encoding the truncated protein was isolated from Btk HD-1, inserted into corn plants and then, through traditional breeding methods, crossed into additional corn lines. The truncated protein is produced within corn and, as demonstrated in greenhouse and field trials conducted by Northrup King Co., protects it from feeding damage by first and second brood European corn borer larvae.

In addition to a gene of Btk HD-1, the corn also carries a gene of the soil microorganism *Streptomyces viridochromogenes*. This gene encodes a selectable marker, the enzyme phosphinothricin-N-acetyl transferase (PAT). The expression of the PAT enzyme enables identification, through the application of the herbicide, glufosinate, of individual corn plants which express the PAT gene and can thus be identified as carrying the Btk gene. The marker is used as a tool in developing inbred lines which are homozygous for the Btk gene. Once the homozygosity is developed, the marker is no longer used.

A great body of scientific evidence accumulated to date, and referenced elsewhere in this petition, demonstrates the specificity toward Lepidopteran insects and the general lack of toxicity of the Btk protein to non-target organisms. The discussions in the following sections review this information, and present additional studies performed by Northrup King Co. and its collaborators, supporting this Petition for Non-Regulated Status.

Additionally, it is expected that significant benefits will be realized as a result of this breakthrough in corn insect pest management. These benefits are reviewed here, in a summary by Prof. Kevin L. Steffey, University of Illinois, Champaign-Urbana, IL:

Agronomic Benefits of Corn Genetically Modified to Resist European Corn Borer and Other Lepidopteran Pests

Prepared by: Kevin L. Steffey, Professor
Office of Agricultural Entomology
University of Illinois
Champaign-Urbana, IL

Executive Summary

Insect protected corn will provide excellent control of an insect (European corn borer) that causes significant decreases in corn yields every year in North America. Currently, corn growers in the eastern Corn Belt treat relatively few acres annually with insecticides to control European corn borers. Corn growers in Colorado, Kansas, and Nebraska treat comparatively more acres to control corn borers. However, yield losses attributable to corn borer damage are appreciable throughout its range. One study in Illinois (Briggs, S.P. & Guse, C.A., 1986. Forty Years of European Corn Borer Data: What Have We Learned? pp 169-173. In: "Thirty-Eighth Illinois Custom Spray Operators Training Manual, Coop. Ext. Serv., Univ. of Ill., Urbana-Champaign.) revealed that approximately 10 percent of the corn acres in that state experience a 9- to 15-percent yield loss annually, attributable solely to the damage caused by the second generation of corn borers. (At least two generations of this pest occur annually throughout most of the Corn Belt.) Results from several studies suggest that corn borers cause an estimated 5 to 7.5 percent yield loss annually (first and second generations combined) (Gray, M. & K. Steffey, 1995. Insect Pest Management for Field and Forage Crops, pp 3-36. In: Pest Management Handbook, Coop. Ext. Serv., Univ. of Ill., Urbana-Champaign; Bergman, P.W., et al, 1985. Pesticide Assessment of Field Corn and Soybeans: Corn Belt States, 26pp, Delta States, 28pp, Lake States, 22pp, Northeastern States, 28pp, Northern Plains States, 22pp; Bode, W.M. & D.D. Calvin, 1990. Yield-loss Relationships and Economic Injury Levels for European Corn Borer (Lepidoptera:Pyralidae) Populations Infesting Pennsylvania Field Corn. *J Econ Entomology* 83(4):1595-1603). These data induce entomologists throughout the United States to consider the European corn borer to be the most under-scouted and under-treated insect that attacks corn. Because European corn borers cause primarily physiological reductions in yield, corn growers are not aware of the significance of their feeding injury during years when infestations are moderate. In addition, efficacy of insecticides applied for control of corn borers is often less than acceptable, particularly for the second generation. Both timing of insecticide applications and placement of the insecticide where corn borer larvae are feeding are difficult. Corn growers frequently are dissatisfied with the level of control of corn borers provided by both chemical and microbial insecticides.

The only other management tactic currently utilized for management of European corn borers is planting of resistant or tolerant corn hybrids. Entomologists and corn breeders have attempted for many years to develop hybrids resistant to European corn borers. However, although some hybrids are resistant to first-generation corn borers, none are resistant to second generation borers. Some hybrids also have the ability to tolerate an infestation of corn borers. Nevertheless, planting of corn hybrids specifically because they are resistant to European corn borers is not widespread, and tolerant hybrids often do not yield as well when infestations of corn borers are heavy.

Insect protected corn promises to be a profound breakthrough in corn insect management. Corn growers who plant insect protected corn will experience yield protection during years when infestations of European corn borers are moderate to large. The potential for substantial reduction or virtual elimination of insecticide use for corn borer control is real. Additionally, the selective activity of the Btk endotoxins will not disrupt populations of either beneficial insects or nontarget animals (e.g., birds, fish). Applications of conventional chemical insecticides often affect nontarget species.

The development of insect protected corn may become a foundation for corn insect management throughout the United States. Reduced insecticide use and improved yields are the likely outcomes of implementation of this technology. If growing insect protected corn effectively eliminates all insecticide applications for European corn borers, corn growers would save a conservative $50 million annually. [This figure was derived from an estimate of 5 percent of the acres of corn treated with insecticides for corn borer control and $15 per acre control costs (insecticide + application costs).] The yield protection benefits gained from controlling corn borer infestations are between 1 and 1.5 billion dollars. (This figure was derived from annual estimates of 70 million acres of corn, an average yield of 120 bushels per acre, an average corn price of $2.35 per bushel and an estimated 5 to 7.5 percent yield loss attributed to corn borer damage.)

The development of insect protected corn will have a major impact on corn pest management. The reduction in the use of aerially applied insecticides will preserve many beneficial insects, and the integration of insect protected corn with other forms of resistance or tolerance will provide solid footing for the development of nonchemical technologies for other major insect pests.

II. Description of the Biology of the Nonmodified Recipient Plant

The Corn Family

Potential for Outcrossing and Weediness of Genetically Modified Insect Protected Corn

Prepared by:

Dr. Arne R. Hallauer
Department of Agronomy, Iowa State University, Ames, IA 50011

Introduction

Corn *(Zea mays* L.), or maize, is one of the few major crop species indigenous to the Western Hemisphere. Corn is grown in nearly all areas of the world and ranks third behind rice *(Oryza sativa* L.) and wheat (*Triticum* sp.) in total production. Corn has been studied extensively, and it seems the probable domestication of corn was in southern Mexico more than 7,000 to 10,000 years ago. The putative parents of corn have not been recovered, but it seems teosinte probably played an important role in the genetic background of corn. The transformation from a wild, weedy species to one dependent on humans for its survival probably evolved over a long period of time by the indigenous inhabitants of the Western Hemisphere. Corn, as we known it today, cannot survive in the wild, because the female inflorescence (the ear) restricts seed dispersal. Although grown extensively throughout the world, corn is not considered a persistent weed nor one difficult to control. A summary of the history, taxonomy, genetics, and life cycle of corn is presented, followed by a discussion of how the characteristics of cultivated corn affect gene flow between cultivated corn and its wild relatives.

1. History of corn

Corn originated in the highlands of Mexico 7,000 to 10,000 years ago. By the time Columbus discovered the Western Hemisphere, corn was being grown by the indigenous civilizations from Chile to southern Canada. Columbus noted the presence of corn on the north coast of Cuba November 5, 1492 and introduced corn to Europe upon his return to Spain (Goodman, 1988). After the introduction of corn to Europe, corn became distributed within two generations throughout the world where it could be cultivated. Today, corn ranks third after wheat and rice as one of the world's three leading food crops. Unlike wheat and rice, more corn is consumed by livestock rather than directly by humans. Corn, however, is consumed directly by humans in the tropics and in the Southern Hemisphere.

The original corn growing areas did not include the north-central area (U.S. Corn Belt) of the United States. The highly productive U.S. Corn Belt dent corns were derived after the colonization of North America. The European settlers accepted the local native American varieties and incorporated them with other crops to provide food, feed, and fuel for their survival. The current U.S. Corn Belt dent corns evolved from the gradual mingling of those settlements that spread north and west from the southeastern North America and those settlements that spread south and west from the northeastern North America.

The corns grown in the northeast are called northern flints; their origin is not clear, but races from the highlands of Guatemala have similar ear morphology (Goodman and Brown, 1988). Northern flints are largely eight-rowed with cylindrical ears, are early maturing, and are short statured plants with tillers. The southern dent corns grown in the southeast United States seemed to have originated from the southeast coast of Mexico. Southern dent corns are characterized as having tall, late maturing, non-tillered, poorly rooted plants with soft textured white kernels on many rowed, tapering ears. It seems the Tuxpeno race contributed to the development of southern dents. The intentional and/or unintentional crossing between the early northern flints and late southern dents led eventually to the highly productive U.S. Corn Belt dent corns that are used extensively throughout the world today.

The origin of corn has been studied extensively, and hypotheses for the origin and for the parentage of corn have been advanced (Mangelsdorf, 1974). Hypotheses suggested for origin of corn include the following: 1) cultivated corn is a descendent of pod corn; 2) corn originated by direct selection from teosinte; 3) corn, teosinte, and *Tripsacum* descended independently from a common, unknown ancestor; and 4) the tripartite theory: a) corn originated from pod corn, b) teosinte derived from a cross of corn and *Tripsacum*, and c) modern corn varieties evolved by corn intercrossing with teosinte or *Tripsacum* or both (Mangelsdorf, 1974).

It has been suggested that modern corn originated from corn grass by a single-gene mutation causing ear development. Other suggestions have included *Coix* and species of the genus *Manisuris* in the tribe *Andropogoneae* for contributing to the genome of corn. The hypotheses have been tested by the study of crosses for genome commonality, fertility, variation, and segregation of morphological plant traits, by archeological evidence, and by use of molecular genetic markers.

Evidence has been reported to support the different hypotheses, but it seems the preponderance of evidence supports the hypothesis that corn descended from teosinte (Galinat, 1988). The teosinte genome is similar to corn, teosinte easily crosses with corn, and teosinte has several plant morphological traits similar to corn. Teosinte has a more weedy appearance and more tillers than modern corn varieties. The one major distinguishing difference between corn and teosinte is the female inflorescence, or ear. Modern corn varieties have 1 to 3 lateral branches that terminate in an ear with 8 to 24 kernel rows of 50 seeds, and the ear is enclosed in modified leaves or husks. Teosinte also has lateral branches, but they terminate in two-rowed spikes of perhaps 12 fruit cases, with each fruit case having one seed enclosed by an indurated glume

(Goodman, 1988).

2. Taxonomy of the genus Zea

Corn is a member of the tribe *Maydae*, which is included in the subfamily *Panicoideae* of the grass family *Gramineae*. The genera included in the tribe *Maydae* include *Zea* and *Tripsacum* in the Western Hemisphere and *Coix, Polytoca, Chionachne, Schlerachne,* and *Trilobachne* in Asia. Although the Asian genera have been implicated by some in the origin of corn, the evidence for them is not as extensive and convincing as for the genera located in the Western Hemisphere.

There has been some fluctuation in Latin binomial designations of the species included in *Zea* in recent years and the classification described by Doebley and Iltis (1980) will be used herein.

The genus *Zea* includes two subgenera: *Luxuriantes* and *Zea*. Corn (*Zea mays* L.) is a separate species within the subgenus *Zea* along with three subspecies. All of the species within the genus *Zea*, except corn, are different species of teosinte. Until recently, the teosinte species were included in the genus *Euchlaena* rather than the genus *Zea*.

The other genus included in the *Maydae* tribe is *Tripsacum. Tripsacum* includes 16 species with a basic set of 18 chromosomes (n = 18), and the different species of *Tripsacum* include multiples of 18 chromosomes ranging from 2n = 36 to 2n = 108.

Five genera are included in the tribe *Maydeae* that originated in Asia. Except for *Coix*, the basic chromosome number is n = 10. Within *Coix*, n = 5 and n = 10 have been reported.

3. Genetics of corn

Corn is genetically one of the best developed and best characterized of the higher plants. Because of the separation of male and female inflorescence, number of seeds produced on female inflorescence, ease in handling (growing and hand pollinating), nature of the chromosomes, and low basic chromosome number (n = 10), corn has been accessible for study at all levels of genetics. Corn was one of the first crop species included in genetic laboratories to obtain a basic understanding of mitosis, meiosis, chromosome segregation, linkage and effects of crossing-over, and transposable elements. Because of the importance of corn in the U.S. and world economies, and the genetic information obtained since 1900, corn has continued to receive extensive study in modern genetic laboratories.

Molecular geneticists have developed extensive genetic maps of corn to complement the genetic maps developed by the early corn geneticists. Corn has been used in tissue culture research, in extensive studies to relate molecular markers to qualitative and quantitative traits, in sequencing of genes, in study of transposable elements for gene tagging and generating genetic variability, in

gene transformation, etc.

Extensive compilations were provided by Coe *et al.* (1988) on corn genetics, by Carlson (1988) on corn cytogenetics, by Phillips *et al.* (1988) on cell tissue culture, and by Walbot and Messing (1988) on molecular genetics. Rapid advances are being made daily in corn genetics, but these are useful references.

4. Life cycle of corn

Corn is an annual and the duration of the life cycle depends on the cultivars and on the environments in which the cultivars are grown (Hanway, 1966). Corn cannot survive temperatures below 0° C (32 F) for more than 6 to 8 hours after the growing point is above ground (5 to 7 leaf stage). Damage from freezing temperatures, however, depends on the extent of temperatures below 0° C, soil condition, residue, length of freezing temperatures, wind movement, relative humidity, and stage of plant development. Light frosts in the late spring of temperate areas can cause leaf burning, but the extent of the injury usually is not great enough to cause permanent damage, although the corn crop will have a ragged appearance because the leaf areas damaged by frost persist until maturity. The completion of the life cycle of corn, therefore, is dictated by the duration of the average number of frost-free days.

The number of frost-free days dictates that corns with differences in length of their life cycles be grown in north-to-south directions of temperate areas. In the United States, corns with relative maturities of 80 days or less are grown in the extreme northern areas, and corns with relative maturities of more than 125 days are grown in the southern areas. Corns having relative maturities of 100 to 115 days are typically grown in the U.S. Corn Belt. Relative maturities, however, are not parallel lines east-to-west because they are dependent on prevailing weather patterns, topography, large bodies of water, and soil types (Troyer, 1994).

Another measure used to judge the relative maturities of corns is the number of growing degree days (GDD) required from emergence to maturity. Based on GDD required to mature, corns are assigned to areas that have, on the average, less than 1850 GDD in the extreme northern areas of the United States to corns that require more than 2750 GDD in more southern areas. Assume a 115-day maturity hybrid is grown in central Iowa. Average last frost date is May 1 and average first frost date is October 5, resulting in an expected 158 frost-free days. If average emergence is May 15 and average flowering is July 15, 60 days are required from emergence to flowering. Corn requires 50 to 60 days to attain physiological maturity. If physiological maturity occurs 55 days after flowering, physiological maturity will occur on or about September 10, or 115 days from emergence to physiological maturity.

If one considers the central U.S. Corn Belt as an example, the following time-frame for each stage of corn development could be as follows:

Planting date:	May 1 ± 10 days
Date emergence:	May 10 ± 4 days
Date of flower:	July 20 ± 10 days
Physiological maturity:	September 10 ± 5 days
Harvest maturity:	October 10 ± 10 days

These suggested time frames can vary within the same year among locations and among years at the same location, depending on the environmental conditions experienced from planting to harvesting.

5. Hybridization

Hybridization is a fundamental concept used in the breeding, production, and growing of corn in the United States. Corn evolved as an open-pollinated (cross-fertilizing) crop species and until the 20th century the corn cultivars were what we designate today as open pollinated corn varieties. Because corn is essentially 100% cross pollinated, the corn varieties were a collection of heterozygous and heterogeneous individuals (genotypes). Varieties were developed by simple mass selection by the indigenous natives prior to the arrival of Columbus. Their methods of selection were simple by present-day standards, but they obviously were effective in developing races, varieties, and strains to satisfy their food, fuel, feed, and cultural needs. Hybridization occurred between varieties as cultures moved within the Western Hemisphere, releasing genetic variability to develop other unique varieties.

The fundamental concepts for development of hybrid corn were defined by 1920 (Sprague, 1946). Basic studies on the genetic composition of a corn variety were conducted to determine the effects of selfing (or inbreeding which is the opposite of outcrossing) within a corn variety (Shull. 1908). Because corn is naturally cross fertilizing, the genetic composition of each plant is not known. Continuous selfing of individuals for 7 to 10 generations resulted in pure lines (or inbred lines) within which every plant had similar traits. The correct interpretation of what occurred during inbreeding was based on Mendelian genetics: the heterozygous loci were eliminated by inbreeding to homozygous loci of either one of the two alleles at each locus. The fixation of alleles in pure lines caused a general reduction in vigor and productivity.

It was found upon crossing two pure lines that vigor was restored. If no selection occurred during inbreeding, the average performance (e.g., grain yield) of all possible crosses was similar to performance of the original variety in which inbreeding was initiated. Some crosses, however, were better than the original open-pollinated variety and could be reproduced from the cross of the pure-line parents of the cross. Hence, the concept of hybrid corn was determined (Shull, 1909): self to develop pure lines, cross the pure lines to produce hybrids, evaluate hybrids to determine the best hybrid, and use of pure-line parents to reproduce the superior hybrid and distribute it for use by the growers.

Hybridization is used in many phases of corn breeding because of the expression of heterosis. Hybridization is used to produce breeding populations (e.g., F2) to develop inbred lines for use in hybrids, and hybridization is used to produce the crosses of superior lines for distribution to growers. Hybridization is easily accomplished either by hand pollinations or by wind pollination in large crossing fields (male and female inbred lines) to produce large quantities of high quality hybrid seed.

6. Potential for outcrossing

a. *Outcrossing with wild Zea species:*

Annual teosinte (2n = 20) and corn (2n = 20) are wind pollinated, tend to outcross, and are highly variable, interfertile species (Wilkes, 1972; 1989). Corn and teosinte are genetically compatible, and in areas of Mexico and Guatemala they freely hybridize when in proximity to each other. Teosinte exists primarily as a weed around the margins of the corn fields, and the frequency of hybrids between teosinte and corn has been studied. Wilkes (1972) reported a frequency of one F1 hybrid (corn x teosinte) for every 500 corn plants or 2 to 5% of the teosinte population for the Chalco region of the Valley of Mexico. As stated by Wilkes (1972), this frequency of hybrids represents a significant gene exchange between a wild weedy plant (i.e., teosinte) and a cultivated relative (i.e., corn). The F1 hybrid of teosinte by corn is robust and fertile and is capable of backcrossing to corn. Intercrossing and gene exchange between teosinte and corn occurs freely, and, accompanied by selection, teosinte had a significant role in the evolution of corn.

Corn easily crosses with teosinte, but teosinte is not present in the U.S. Corn Belt. The natural distribution of teosinte is limited to the seasonally dry, subtropical zone with summer rain along the western escarpment of Mexico and Guatemala and the Central Plateau of Mexico (Wilkes, 1972). Except for special plantings, teosinte is not found in the United States, and there have been no instances reported that teosinte occurs as a weed along the margins of corn plantings in the U.S. Corn Belt.

Tripsacum-corn hybrids have not been observed in the field and *Tripsacum*-teosinte hybrids have not been produced (Wilkes, 1972). *Tripsacum* evolved by polyploidy, whereas corn and teosinte have undergone introgressive hybridization at the diploid level (2n = 20). The diploid forms of *Tripsacum* (2n = 36) are morphologically distinct and allopathic in their distribution (Wilkes, 1989). *Tripsacum* species are perennials and seem to be more closely related to the genus *Manisuris* than to either corn or teosinte (Goodman, 1976). *Tripsacum* received greater interest in the evolution of corn after Mangelsdorf and Reeves (1931) successfully crossed corn and *Tripsacum dactyloides* (2n = 36). The cross by Mangelsdorf and Reeves (1931) was made with the diploid *Tripsacum dactyloides* (2n = 36) as the male parent. Silks of the female corn parent were cut to permit successful pollination. The cross had 28 chromosomes and was male sterile. Five other *Tripsacum* species have been crossed with corn, and Galinat (1988) has mapped more

than 50 homologous loci on the chromosomes of corn and *Tripsacum*. In contrast with corn and teosinte being easily hybridized, both in the wild and by controlled pollinations, it requires special techniques to hybridize corn and *Tripsacum*. Except for *Tripsacum floridanum*, it is difficult to cross *Tripsacum* with corn, and the offspring of the cross show varying levels of sterility. Small portions of *Tripsacum* genome can be incorporated by backcrossing.

Sixteen species of *Tripsacum* have been described. *Tripsacum floridanum* is native to southern tip of Florida. Twelve of 16 *Tripsacum* are native to Mexico and Guatemala. *Tripsacum australe* and two other species are native to South America. The center of variation for *Tripsacum* is the western slopes of Mexico, the same area where teosinte is frequently found. The habitat preferences of *Tripsacum* are similar to those for teosinte: seasonally dry, summer rains, elevation of 1600 m, and limestone soils (Wilkes, 1972).

b. Outcrossing with cultivated Zea varieties:

Corn is wind pollinated, and the distances that viable pollen can travel depend on prevailing wind patterns, humidity, and temperature. Occasionally it has been found that corn pollen can travel up to 3.2 km (2 miles) by wind under favorable conditions. All corns will interpollinate, except for certain popcorn varieties and hybrids that have one of the gametophyte factors (Gas, Ga, and ga allelic series on chromosome 4). Pollen of a specific hybrid can be carried by wind to pollinate other dent corn hybrids, sweet corn, and popcorn, if the popcorn does not carry the dent-sterile gametophyte factor. Corn pollen, therefore, moves freely within an area, lands on silks of the same cultivar or different cultivars, germinates almost immediately after pollination, and within 24 hours completes fertilization. Although there may be some minor differences in rate of pollen germination and pollen tube elongation on some genotypes, corn pollen is very promiscuous. It is estimated each corn plant can shed more than 10 million pollen grains.

Certification standards for distances between different corn genotypes have been established to assist in the production of hybrid corn having desired levels of purity. A specific isolation field to produce commercial hybrid seed shall be located so that the seed parent is no less than 200 m (640 feet or 40 rods) from other corn of a similar type; i.e., if seed parent is a yellow, dent corn it should be isolated at least 200 m from other yellow, dent corns. The distance of 200 m can be modified because of size of field, number of border rows, and different maturity dates of flower, provided no receptive silks are available at the time pollen is being shed from the contaminating field. If the hybrid seed being produced is of a different color or texture from neighboring contaminating fields, the distances and the number of border rows should be increased.

7. Weediness of corn

Modern-day corn cannot survive as a weed. One does not find volunteer corn growing in fence rows, ditches, and road sides as a weed. Although corn from the previous crop year can overwinter and germinate the following year, they cannot persist as a weed. The appearance of

corn in soybean fields following the corn crop from the previous year is a common occurrence. Measures are often taken to either eliminate the plants with the hoe or use of herbicides to kill the plants in soybean fields, but the plants that remain and produce seed usually do not persist the following years.

It is difficult for the corn to survive as a weed because of past selection in the evolution of corn. In contrast with weedy plants, corn has a polystichous female inflorescence (or ear) on a stiff central spike (or cob) enclosed with husks (modified leaves). Consequently, seed dispersal of individual kernels naturally does not occur because of the structure of the ears of corn. Individual kernels of corn, however, are distributed in fields and main avenues of travel from the field operations of harvesting the crop and transporting the grain from the harvested fields to storage facilities. In neither instance (natural or mechanical harvesting) does corn become a troublesome weed. Corn cannot survive without human assistance and is not capable of surviving as a weed.

Environmental Consequences Of Introduction Of The Transformed Variety

1. Weediness of a transformed corn variety

In the past 10 years, techniques have been developed for gene transfer into plants. Gene transformation is the acquisition by a cell of new gene(s) by the uptake of naked DNA, which can be by direct introduction of DNA and by either the Ti system or through protoplast transformations. Two of the more common applications of gene transfer being used in corn are the introduction of gene(s) conferring insect resistance or herbicide tolerance. Herbicide tolerance is usually conferred by single genes that interact with key enzymes in important metabolic pathways. Insect resistance is typically conferred by expression of the *Bacillus thuringiensis* (Bt) protein. The lines and hybrids that include the transferred gene(s) (e.g., herbicide tolerance and insect resistance) will have to meet the standards of non-transformed lines and hybrids to be competitive in the marketing of hybrid seed corn. The introduction of genes by the newer molecular techniques will be more precise than the classical backcross methods and will be directed primarily to single genes. The overall phenotype of transformed plants will be very similar to the original phenotype: the reproductive organs (tassels and ears), duration of plant development, methods of propagation, ability to survive as a weed, etc. will not change.

2. Potential for outcrossing of the transformed variety

a. *Outcrossing with wild Zea species:*

Outcrossing of transformed corn plants with wild relatives of corn will be the same as for non-

transformed corn plants. Outcrossing with teosinte species will only occur where teosinte is present in Mexico and Guatemala. Outcrossing with *Tripsacum* species is not known to occur in the wild and only under very carefully conditions can corn be crossed with *Tripsacum*. In the United States, only *Tripsacum floridanum* is known to be present in southern tip of Florida. Teosinte and *Tripsacum* are included in botanical gardens in the United States and the possibility exists, though unlikely, that the exchange of genes would occur between corn and its wild relatives. No cases of gene flow between corn and its wild relatives are known in the United States.

b. *Outcrossing with cultivated Zea varieties:*

Gene exchange between cultivated corn and transformed corn would be similar to what naturally occurs at the present time. Windblown pollen would move about among plants within the same field and among plants in nearby fields. Free flow of genes would occur similar to what occurs in cultivated corn. The transformed plants include individual genes and depending on the relative expression of the transformed genes (relative levels of dominance for gene expression), plant architecture, and reproductive capacities of the intercrossed plants will be similar to normal corn. The chances that a weedy type of corn will result from outcrossing with cultivated corn is extremely remote.

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III. Relevant Experimental Data

The following studies are submitted in support of this Petition for Non-Regulated Status:

Study A: **Analysis of Effects of Btk Conversion on Phenotypic and Yield Attributes of *Zea mays* L.**

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Northrup King Co., Stanton, MN

The original transformation event which gave rise to all Btk-converted germplasm which is the subject of this petition has been designated 'Bt11' within Northrup King Co. The genetic background, coded BG, was the recipient of the Btk construct in this transformation event. All subsequently developed genetic material traces back to a single plant for which this particular event placed the Btk construct at a specific site on chromosome 8 in the genetic background of BG.

Four generations of backcrossing (to BC_4), supplemented by selection, were used to transfer the chromosome fragment containing the Btk coding sequence into the genetic background of elite maize inbred lines. A series of three independent conversions of the inbred 2043 were derived through parallel backcrossing schemes. Likewise, two independent conversions of the inbred, 2044, were used to obtain a Btk version of the original 2044. These BC_4 derivatives were then subjected to extensive experimental analysis to assure that they represented adequate recoveries of the essential features of the original elite lines. The evaluation process was as follows:

The two 2044 conversions, along with the original 2044, were crossed to a series of five, unrelated, elite inbred lines to produce F_1 hybrid seed for evaluation in replicated trials in 1994. Eighteen hybrids were produced for evaluation. Evaluation consisted of planting each hybrid at each of 17 locations which were distributed across 8 states and 1 Canadian providence. The trial design at each site was a randomized complete block design with two replicates. Each plot was represented by two rows (paired) of 20 feet in length, including three foot alleys. The plant population varied within the range of 25,000 to 27,000 plants per acre at each location. Notes and measurements were taken on the plots during the growing season, as is usual for commercial variety trials. At harvest, a grain combine, which is adapted to harvest of corn plot trials, was used to determine plot yield and grain moisture at harvest. Variables which were measured, (and the number of locations at which each trait was evaluated) include: Yield (18), Grain Moisture (18), Stalk Lodging Rating (14), Root Lodging Rating (14), Ear Height (4), Plant Height (4), Heat Units to Silking (3), Heat Units to Pollen Shed (3), and Intactness Rating (1). Data was subjected to a factorial analysis of variance using the SAS GLM Procedure.

The model for analysis was:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\beta\gamma)_{jk} + \epsilon_{ijkl}$$

where μ is the grand mean, α_i is the effect of the i^{th} location, β_j is the effect of the j^{th} inbred used

in testcrossing, γ_k is the effect of the k^{th} Btk conversion line of the inbred 2044, etc. A single degree of freedom contrast was used to test the significance of the difference between the original, non-transformed, 2044 line effects and those of the transformed derivatives.

An identical process was used in testing the Btk conversions of the elite inbred, 2043, except that there were three independent backcross conversions, rather than two. Btk-converted lines were tested in combination with 6 elite inbreds and resulting hybrids were evaluated in 17 locations. The number of locations of data for each trait are as follows: Yield (17), Grain Moisture (17), Stalk Lodging Rating (9), Root Lodging Rating (11), Ear Height (2), Plant Height (2), Heat Units to Silking (1), Heat Units to Pollen Shed (1), and Intactness Rating (5).

Results of analysis of variance are presented in Table A1. Two statistics are presented for each trait. The first is the significance level of the contrast which compares the original 2044 (or 2043) line effects to the average of the two backcross derivatives which contained the Btk sequence. The second is the significance level of the test which compares the effects of the original 2044 (or 2043) line to the specific backcross derivative which was selected based upon best phenotypic conformity to the original line. Also listed are the trait performances (Least-squares Means) for the original and selected Btk conversion, averaged across all testers and locations.

Significant differences were observed between the original elite line 2044 and the Btk conversions for the traits: Stalk Lodging, Heat Units to Silking, Heat Units to Pollen Shed, and Intactness Rating. Similarly for 2043 and its derivatives, differences were observed for Moisture, Stalk Lodging and Intactness Rating. The selected Btk conversion lines were different from the original, non-transformed parents only for Stalk Lodging Rating and Intactness Rating in the case of both 2044 and 2043 conversions. Both of these traits are expected to be affected by corn borer damage. Thus, results are encouraging, but not surprising. The "disappearance" of significant effects for Heat Units to Silking and Pollen Shed and for Moisture at Harvest are likely to be due to superior recovery of the original genome of the elite lines in the selected Btk conversion as compared to the unselected conversions. This is a likely explanation because the donor genome which carried the Btk coding sequence was a very early-maturing line which would be expected to affect Heat Units to Flowering and Harvest Moisture if its genes were not eliminated during the backcrossing process.

No differences were observed between hybrids derived using original elite lines and the selected Btk conversion lines for any of the traits: Yield, Moisture at Harvest, Root Lodging Rating, Ear Height, Plant Height or Heat Units to Silking or Pollen Shed. This result indicates that the selected Btk conversion lines are good recoveries of the original genome of the elite lines for chromosome segments other than the single region encoding Btk. At least, other <u>important</u> regions must be well-recovered. Secondarily, these results indicate that the insertion of the Btk sequence does not alter plant morphology or agronomic performance for traits unrelated to the Btk function.

Table A1. The effects associated with Btk backcross conversions in inbred backgrounds of the elite lines 2044 and 2043 based on the significance of single degree-of-freedom contrasts comparing elite line with 1) the average of all BC_4 derivatives and 2) the selected conversions. Also presented is the average performance (Least-squares Means) of the original elite line and the selected Btk conversion line in testcross performance with multiple testers and across locations.

2044 Conversions

Trait	Normal vs Btk	Normal vs Selected Btk
Yield (Bu/A)	NS[1]	NS (180.4 vs 183.4)
Moisture (%)	NS	NS (17.99 vs 18.29)
Stalk Lodging[2]	***	*** (2.04 vs 1.07)
Root Lodging[2]	NS	NS (1.48 vs 1.29)
Ear Height (cm)	NS	NS (88.38 vs 80.85)
Plant Height (cm)	NS	NS (257.5 vs 253.2)
H.U. to Silk	*	NS (1342 vs 1341)
H.U. to Pollen	*	NS (1328 vs 1329)
Intactness Rating[2]	**	* (4.10 vs 3.10)

2043 Conversions

Trait	Normal vs Bt	Normal vs Selected Bt
Yield (Bu/A)	NS	NS (188.73 vs 190.84)
Moisture (%)	*	NS (18.94 vs 18.74)
Stalk Lodging[2]	***	* (2.87 vs 1.76)
Root Lodging[2]	NS	NS (2.89 vs 4.41)
Ear Height (cm)	NS	NS (112.9 vs 117.3)
Plant Height (cm)	NS	NS (282.7 vs 284.7)
H.U. to Silk	NS	NS (1385 vs 1387)
H.U. to Pollen	NS	NS (1358 vs 1356)
Intactness Rating[2]	***	** (4.80 vs 3.18)

[1] NS, *, **, and *** indicate non-significance, and significance at the 0.05, 0.01 and 0.001 levels of probability, respectively.

[2] lower values for ratings indicate superior performance.

Study B: **Southwestern Corn Borer, *Diatraea grandiosella*, and Fall Armyworm, *Spodoptera frugiperda*, versus Btk-Maize Hybrids.**

Authors:
Jonathan B. Sagers and David W. Mies
Northrup King Co., Stanton, MN
Paul Williams and Frank Davis
USDA-ARS, Starkville, MN

1994 marked the first of a two-year cooperative effort with the USDA-ARS to assess the effectiveness of Btk-maize hybrids to the southwestern corn borer (SWCB) and the fall armyworm (FAW).

Materials and Methods:

Dr. Paul Williams and Dr. Frank Davis, both of USDA-ARS, Starkville, MS, used standard methods of artificial infestation and subsequent evaluation to assess control of these insect pests of corn. Each insect species was tested in a separate trial and four replications were planted. They artificially infested plants with approximately 30 insects per plant. FAW larvae were allowed to feed for seven days, then plant damage ratings (0-9) were assigned. The rating scale is one developed by Williams, W.P., et al (1983. Fall Armyworm Resistance in Corn and Its Suppression of Larval Survival and Growth. *Agron J* 75:831-832). A zero rating represented no damage and possibly a plant that was not infested (escape). A one rating represented pin-hole sized feeding damage on a few leaves, while a nine represented a large area of leaf tissue removed from several leaves. On the tenth day post-infestation, plants were dissected and all living larvae were recovered from each plant. These were collected, counted, and weighed. Then the average weight per insect and the average biomass of insects per infested plant were calculated.

Additional plants were grown to supply leaf tissue for use in laboratory feeding assays. Leaf whorl tissue was frozen, lyophilized, ground, and incorporated into agar, then fed to fall armyworm and southwestern corn borer larvae according to the methods of Buckle, et al (Buckle, P.M., F. M. Davis, and W.P. Williams. 1991. Identifying Resistance in Corn to Corn Earworm (Lepidoptera: Noctuidae) Using a Laboratory Bioassay. *J. Agric. Entomol.* 8:67-70).

In southwestern corn borer field trials, larvae were allowed to feed for fourteen days, then plants were evaluated for leaf feeding damage as described above. Plants were dissected and live larvae were counted, collected and weighed. The same calculations as described for FAW were performed and data were analyzed. Analysis of variance calculations were performed to analyze these data.

Results:

See the attached tables for results of these experiments. Table B1 and Figure B1 display 1994 field results against FAW. Figure B2 and Table B2 display SWCB results. Table B3 entitled: "Fall Armyworm and Southwestern Corn Borer Larval Growth on Lyophilized Leaf Tissue in a Laboratory Bioassay" shows mean larval weights of larvae allowed to feed on lyophilized leaf whorl tissue in laboratory diet assays.

All three parameters measured (FAW leaf feeding damage, larval survival, and biomass per plant) were significantly reduced on all Btk hybrids tested except BT8 (see Table B1). These Btk hybrids provided control comparable to but not significantly different from sources of resistance developed by Williams and Davis using traditional breeding methodology. However, efficacy was significantly improved over non-Btk control NK hybrids.

In SWCB tests, all Btk hybrids including BT8 demonstrated significantly reduced leaf feeding damage compared to sources of resistance developed through traditional breeding (see Table B2). The number of surviving larvae and the average biomass per plant were also significantly reduced relative to susceptible non-Btk hybrids and susceptible standard checks. Larval survival and biomass per plant compared favorably with traditionally developed resistant controls. The average number of surviving larvae was significantly lower than traditional resistance sources on all Btk hybrids with the exception of BT6.

Results shown on the table of laboratory assay results indicated that SWCB larvae were unable to survive on Btk hybrid leaf tissues, while the FAW did survive fairly well. In some Btk hybrids, the larval survival may have been reduced significantly relative to traditional resistant controls, but at least 50% of the larvae did survive. Mean larval weights indicated that Btk hybrids did significantly reduce larval weights relative to traditional sources of resistance.

Conclusions:

Btk hybrids show excellent potential for assisting in the control of FAW and SWCB. Btk hybrids produced using event Bt11 provided better control of FAW than hybrids transformed using event Bt10. Both events appeared to provide significant control of SWCB. Hybrid "BT8" was developed using a different Btk transformation event designated as "Bt10". Through previous research it is known to produce lower levels of Btk protein within the plant tissues than Bt11, the other event evaluated in these trials. Hybrids BT1 - BT7 were developed using event Bt11. Tissue samples were collected just prior to tassel emergence of several hundred Bt10 and Bt11 hybrids. ELISA (enzyme-linked immunosorbant assay) was performed on them to assess protein levels. Bt10 protein levels were very low (data not shown) and in many cases were not detectable. The late stage of plant growth when tissues were sampled may have resulted in reduced levels of detectable protein. However Bt11 hybrids typically produced higher levels of Btk protein, which apparently was important in providing good control of FAW. Therefore, SWCB appears to be more susceptible to Btk toxin than the FAW.

In Btk hybrid designated BT6 in these trials, seed purity was a problem, and ELISA results indicated that several plants displaying damage were not expressing Btk protein. However, larval data collected from Btk negative plants are included in the results, so the average SWCB "larvae/ plant" of 1.4 shown on Table B2 may be artificially inflated due to inclusion of Btk-negative plants.

Table B1. Evaluation of transgenic and other corn lines for reaction to fall armyworm.

Entry	Leaf Feeding Rating [1]	Larvae / Plant [2] (no.)	Biomass / Plant [3] (mg)
BT1	1.4	0.1	3
BT2	1.1	0.0	2
BT3	1.0	0.0	0
BT4	1.3	0.1	1
BT5	1.0	0.0	0
BT6	1.4	0.1	13
BT7	1.1	0.0	0
BT8	3.0	1.1	56
NK1	4.0	1.5	82
NK2	2.9	1.0	51
NK3	3.4	1.0	55
NK4	4.0	1.4	66
NK5	3.6	0.8	53
NK6	4.1	1.3	94
Mp704xMp707(R)[4]	1.4	0.1	6
Mp704xMp708(R)	2.2	0.1	1
Ab24ExSC229(S)	5.5	2.0	165
SC229xTx601(S)	4.6	1.9	149
LSD (0.05)	0.8	0.6	45

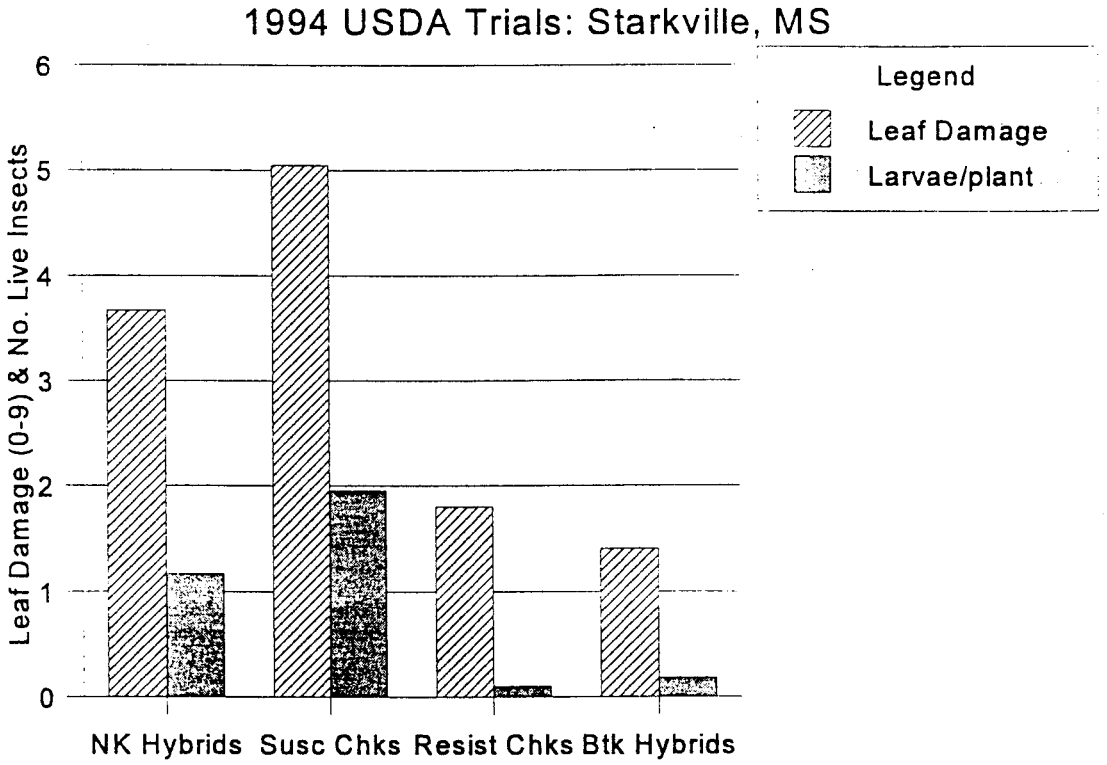
[1] Fall armyworm leaf feeding damage rated 7 days after infestation with 30 larvae/plant on a scale of 0 (no damage) to 9 (extensive damage).

[2] Mean number of larvae/plant calculated by dividing the number of larvae recovered in a plot by the number of plants (usually ten).

[3] Biomass/plant determined by adding weights of all larvae from a plot and dividing by number of plants.

[4] R indicates resistant check; S indicates susceptible check.

Figure B1: Fall Armyworm Damage



Leaf damage based on 0-9 damage rating scale of Williams *et al.* (1983). NK Hybrids = Average of 6 hybrids. Susc Chks = Average of 2 susceptible check hybrids. Resist Chks = Average of 2 most resistant hybrids. Btk Hybrids = Average of 8 Btk hybrids. (Leaf Damage $LSD_{(a=0.05)} = 0.8$). (# Larvae/Plant $LSD_{(a=0.05)} = 0.6$).

Table B2. Evaluation of Transgenic and Other Corn Lines for Reaction to Southwestern Corn Borer.

Entry	Leaf Feeding Rating [1]	Larvae / Plant [2] (no.)	Biomass / Plant [3] (mg)
BT1	1.5	0.3	20
BT2	2.7	0.4	17
BT3	1.2	0.0	0
BT4	1.1	0.1	2
BT5	1.0	0.0	2
BT6	1.5	1.4	26
BT7	1.1	0.0	0
BT8	1.5	0.1	5
NK1	6.7	6.1	378
NK2	6.9	6.0	380
NK3	7.0	5.2	313
NK4	6.7	5.1	287
NK5	6.6	5.6	339
NK6	6.7	5.7	364
Mp704xMp707(R)[4]	4.2	1.8	10
Mp704xMp708(R)	4.7	1.9	23
Ab24ExSC229(S)	7.3	5.5	371
SC229xTx601(S)	6.8	4.5	234
LSD (0.05)	1.2	1.4	69

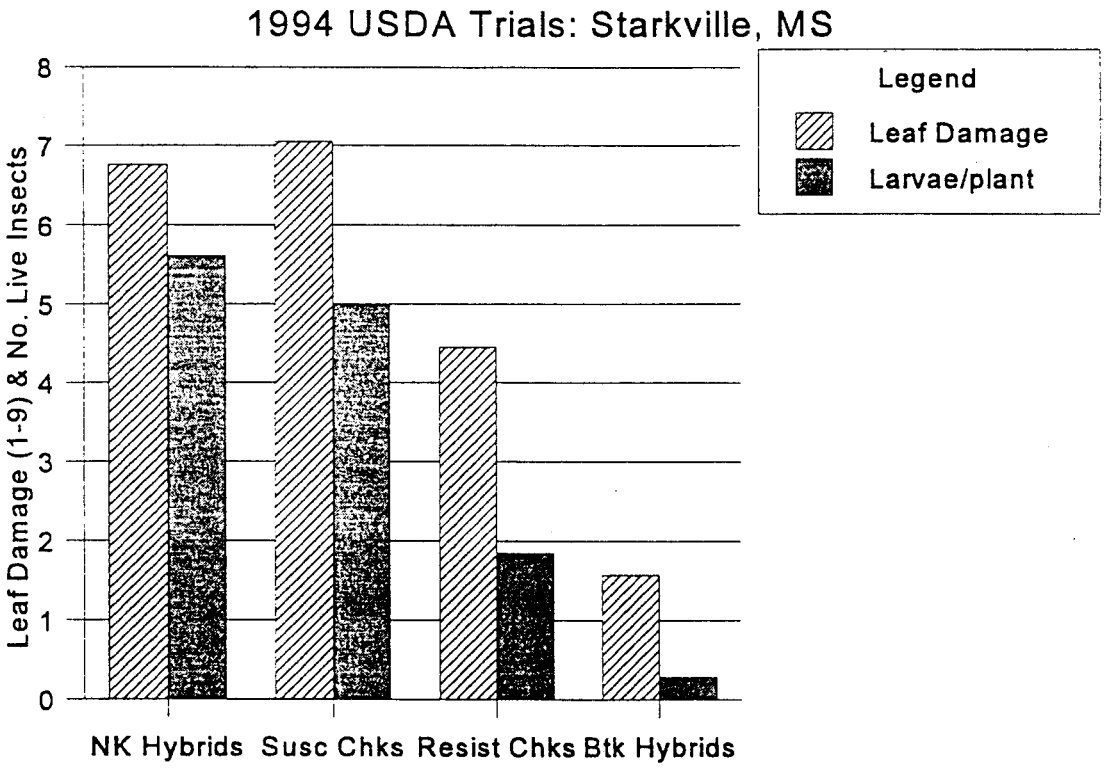
[1] Southwestern corn borer leaf damage visually rated 13 days after infestation with 30 larvae/plant on a scale of 0 (no damage) to 9 (extensive damage).

[2] Mean number of larvae/plant calculated by dividing the number of larvae recovered in a plot by the number of plants (usually ten).

[3] Biomass/plant determined by adding weights of all larvae from a plant and dividing by number of plants.

[4] R indicates resistant check; S indicates susceptible check.

Fig. B2: Southwestern Corn Borer Damage



Leaf damage based on 1-9 ECB damage rating scale of Guthrie *et al.* (1960). NK Hybrids = Average of 6 non-Btk hybrids. Susc Chks = Average of 2 susceptible control hybrids. Resist Chks = Average of 2 most resistant hybrid controls. Btk Hybrids = Average of 8 Btk hybrids. (Leaf Damage $LSD_{(a=0.05)} = 1.2$). (# Larvae/Plant $LSD_{(a=0.05)} = 1.4$).

Table B3. Fall Armyworm and Southwestern Corn Borer Larval Growth on Lyophilized Leaf Tissue in a Laboratory Bioassay.

Genotype	Larval Survival (%)		Mean Larval Weights (mg)	
	FAW	SWCB	FAW [1]	SWCB [1]
SC229xTx601	100	95	176	91
Mp704xMp708	100	95	102	37
NK4	95	85	341	47
NK5	90	90	300	37
NK3	90	40	309	23
Mp704xMp707 [4]	90	75	112	34
NK6	90	75	285	40
Ab24ExSC229 [2]	85	80	400	86
NK2	85	90	124	106
BT8	80	-- [3]	93	-- [3]
BT2	80	0	11	--
BT6	70	0	12	--
BT5	65	0	8	--
BT7	65	0	13	--
BT4	65	0	38	--
NK1	60	55	334	20
BT3	60	0	8	--
BT1	50	0	28	--
LSD (0.05)	23	20	51	15

[1] FAW larvae weighed after 10 days; SWCB larvae after 14 days.

[2] Susceptible check.

[3] BT8 was not included in the SWCB bioassay.

[4] Resistant check.

Study C: Northrup King Co. Btk-Maize versus the Fall Armyworm, *Spodoptera frugiperda*

Authors:
Jonathan B. Sagers and David W. Mies
Northrup King Co., Stanton, MN
Paul Williams and Frank Davis
USDA-ARS, Starkville, MN

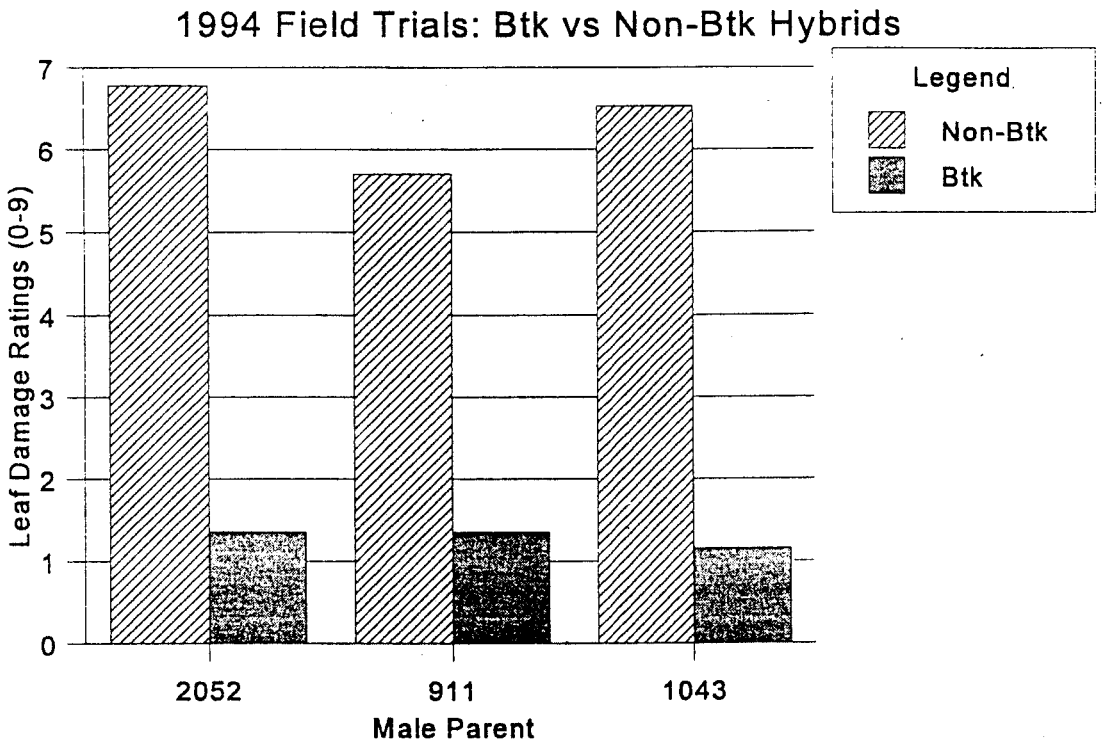
Experimental Design:

These trials contained three Btk maize hybrids and three similar non-Btk control hybrids. Plants were grown at the same locations and under the same agronomic practices as the CEW trials. Plants were artificially infested fairly late in development at approximately the 11th to 12th leaf stage of development. Plants were grown in two-row plots and only plants of the first row of each plot were infested. This created a one-row uninfested buffer between each infested row, and minimized migration of large larvae from susceptible control plants to suspected resistant plants. The fall armyworm colony was obtained from Dr. Frank Davis, USDA-ARS Entomologist, Starkville, MS. An average of 27 insects were applied to each infested plant. Ten plants were infested per row. Trials were evaluated for leaf feeding damage ratings after 18 days. Plant damage was assessed using a 0-9 damage scale developed by Williams, W.P., et al (1983. Fall Armyworm Resistance in Corn and Its Suppression of Larval Survival and Growth. *Agron J* 75:831-832) on which 0 = no damage, 1 = small pinholes on a few leaves, and 9 = large portions of leaf eaten from several leaves. Live larvae were collected and counted from either five or ten plants per infested row. They were refrigerated and weighed in the lab the day following collection. The following data were generated: 1) counts of live larvae recovered per artificially infested plant, 2) average individual larval weight per treatment, and 3) the average biomass of live larvae per artificially infested plant.

Results:

Figure C1 shows that the average leaf damage rating on Btk hybrids was approximately 1.2. This compared to an approximate average of 6.2 on the non-Btk control plants. Table C1 displays the differences in larval counts, weights and biomass per plant between Btk and non-Btk corn plants.

Figure C1: Fall Armyworm Damage



Leaf damage rating of Williams et al. (1983), where 0 = uninfested plant (escape), 1 = pinhole damage only, 2 = shot hole damage on a few leaves, 3 = shot hole feeding damage on several leaves...7 = large lesions on and portions of leaves eaten away on a few leaves...9 = large portions of leaf eaten away on most of plant. Trials were conducted under artificial infestation at Stanton and Kenyon, MN. ($LSD_{(a=0.05)} = 0.28^{**}$).

Table C1. Fall Armyworm Growth and Survival on Btk Corn.

Hybrid	Avg. No. Live Larvae Recovered Per Plant	Avg. Weight of Survivor (mg)	Avg. Biomass of Larvae per Plant (mg)
2052 Btk	0.05	10.0	0.6
2052 Non-Btk	2.90	80.0	153.0
911 Btk	0.05	10.0	0.5
911 Non-Btk	2.75	60.0	136.0
1043 Btk	0.23	20.0	5.5
1043 Non-Btk	4.08	110.0	264.0
LSD (a = 0.05)	0.37	20.0	84.6

Summary and Conclusions:

Dramatic and significant differences were demonstrated in all measured parameters of the interaction between Btk maize hybrids, non-Btk maize hybrids, and FAW larvae. Leaf damage ratings, larval survival, average larval weights, and larval biomass per plant were all significantly reduced on Btk maize versus non-Btk maize.

Initial first-year results indicated the Btk event expressed in these hybrids substantially reduced the survival of FAW on maize. This subsequently reduced the amount of leaf damage caused by these insects. One concern in these trials, however, was that the insects used to artificially infest plants may have been diseased or less vigorous than true wild type insects. Dr. Frank Davis, USDA-ARS Research Entomologist, and Dr. Paul Williams, USDA-ARS Supervisory Research Geneticist, Starkville, MS, conducted cooperative trials with the FAW as well. In their trials, standard susceptible control plants normally included in all FAW tests were not damaged by FAW of this colony as severely as in normal years. Therefore, first year results must be confirmed in second year trials. These confirmation trials are currently underway at Starkville, MS, (USDA-ARS) and at Kenyon and Stanton, MN.

Study D: **Northrup King Co. Btk-Maize versus the Corn Earworm, *Helicoverpa zea***

Authors:
Jonathan B. Sagers and David W. Mies
Northrup King Co., Stanton, MN

Experimental Design:

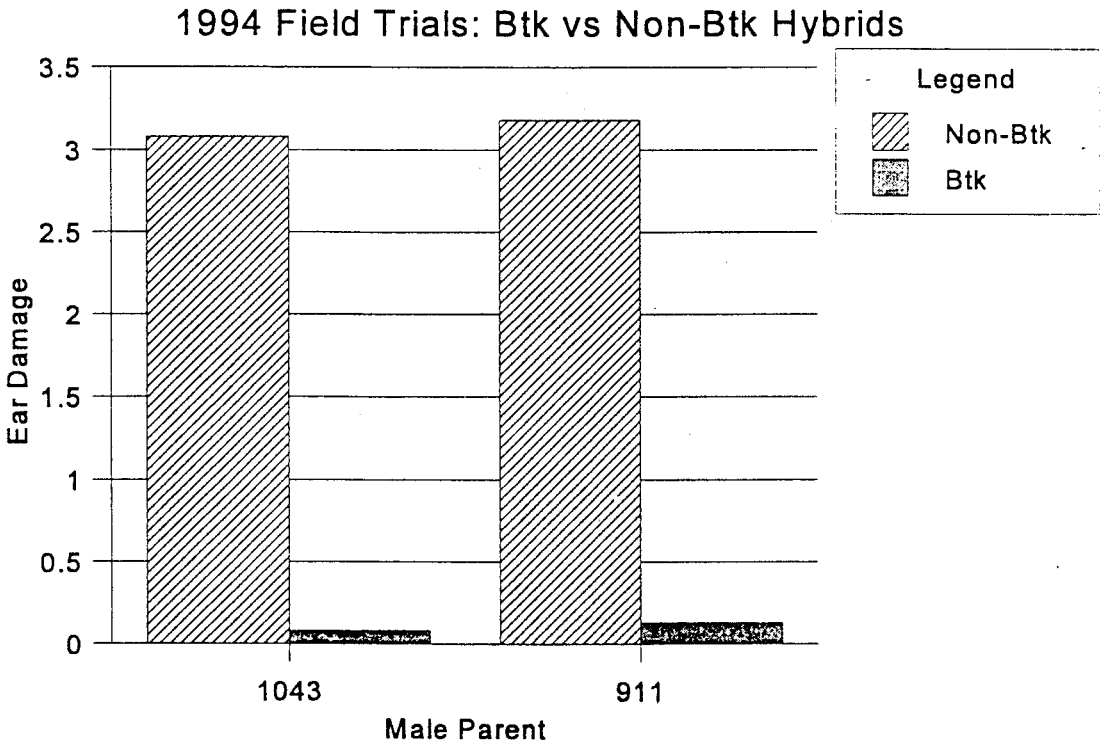
Small trials of Btk maize hybrid corn were planted at Kenyon, MN and Stanton, MN. The trials contained two Btk maize hybrids and representative non-Btk hybrid controls. Trial design was randomized complete block with two replications per test location. Plants were grown under standard agronomic practices for this geographic area. Each plant was artificially infested with 20 eggs of corn earworm (CEW) following emergence of green silks. The eggs were purchased through French Agricultural Research, Inc., Lamberton, MN. Eggs were suspended in dilute agar solution and injected into the silk channel of each ear. Plants with adequate silk emergence were chosen at random for infestation. Labels were permanently affixed to infested plants to identify them as infested.

Larvae were allowed to feed and develop for 29 days (Kenyon, MN) and 30 days (Stanton, MN). By 29-30 days post-infestation, most larvae on susceptible control plants had matured and left the plants, but damage to the kernels was still very easily measured. Larvae were still present on nineteen of one hundred sixty (160) ears evaluated, so diagnosis and confirmation that this damage was caused by CEW was easily confirmed. Ear and kernel damage was evaluated using the scale of Widstrom, N.W., et al (1967. An Evaluation of Methods for Measuring Corn Earworm Injury. *J Econ Entomol* 60:791-794). On this scale, "0" represents no damage, "1" represents silk clipping only, "2" represents one centimeter of kernel damage at the tip of the ear, "3" represents two cm damage, and so on.

Results, Summary and Conclusions:

As shown in Figure D1, two non-Btk hybrids sustained significantly more corn earworm feeding damage than Btk hybrids. Eighty Btk ears were evaluated, and only three displayed damage to the kernels. In contrast, seventy-five of eighty non-Btk ears were damaged. The average damage ratings for the two Btk maize hybrids were 0.13 and 0.08. The average damage ratings for non-Btk hybrid ears were 3.08 and 3.18 ($LSD_{(a=0.05)} = 0.95^{**}$). Thus, on average the negative control hybrids displayed approximately 2.0 cm of kernels at the tip of each ear damaged by CEW, while the Btk maize hybrids demonstrated very limited silk clipping but virtually no kernel damage. Therefore, based on this first-year study, it appears that these Btk maize hybrids display high levels of resistance versus the corn earworm under artificially infested field conditions. Trials will be repeated in 1995 at the same sites to verify 1994 field results.

Figure D1: Corn Earworm Damage



Ear Damage Rating Scale: 0 = no damage, 1 = silk feeding only, 2 = 1 cm kernel feeding damage, 3 = 2 cm kernel feeding damage...(according to Widstrom, 1967). Trials were conducted under artificial infestation at Stanton and Kenyon, MN. ($LSD_{(a=0.05)}$ = 0.95**).

Study E: **Northrup King Co. Btk-Hybrid Maize versus Artificially Infested European Corn Borer - 1994 Field Trials**

Authors:
Jonathan B. Sagers and David W. Mies
Northrup King Co., Stanton, MN

Introduction:

Throughout the US, the European corn borer (ECB) causes heavy losses in corn production through plant damage and associated yield and grain quality reduction each year. We have conducted research under field conditions to evaluate Btk hybrid corn's effectiveness against the European corn borer. Results for the past three years have indicated Btk corn effectively controls damage of the ECB.

Material and Methods:

Trials were conducted using a randomized complete block design. Two replicates were planted at three locations across three states in two-row plots. Hybrids were grouped according to relative maturity and planted at appropriate sites based on maturity. The northern adapted hybrids were planted at Janesville, WI, Stanton, MN, and Webster City, IA. Southern adapted hybrids were grown at St. Joseph, IL, Washington, IA, and Phillips, NE. The southern trials contained six Btk hybrids and four non-Btk control hybrids. The northern trials consisted of eight Btk hybrids and two non-Btk control hybrids. Plants were artificially infested as they approached the V6 stage of growth. Approximately fifty larvae were applied to ten plants in the first row of each plot every three to four days over a two and one-half week period. By the end of first generation infesting, each plant had been infested with at least 200 neonate larvae. Just prior to tassel emergence, 1-9 leaf damage ratings were assigned to each of the ten plants per plot. The rating scale of Guthrie, W.D., et al. (1960. Leaf and Sheath Feeding Resistance to the European Corn Borer in Eight Inbred Lines of Dent Corn. *Ohio Ag Exp Sta Res Bulletin* 860) was used, where 1 = no damage or a few pinholes, 2 = small holes on a few leaves, 3 = shot-holes on several leaves, 4 = irregular shaped holes on a few leaves... 9 = several leaves with many merging elongated lesions.

As plants began to shed pollen, second generation ECB infesting began. Once again the first ten plants of the first row of each plot was infested with 40-50 larvae every three to four days over a two and one-half week period. By the end every plant had been infested with approximately 200 or more larvae. After approximately 45-50 days, plants were dissected from the top to the ground and the total length of tunnels created by ECB feeding was measured (estimated) in inches. This was converted to centimeters for reporting. Analysis of Variance and Least Significant Difference mean separation were used to analyze results.

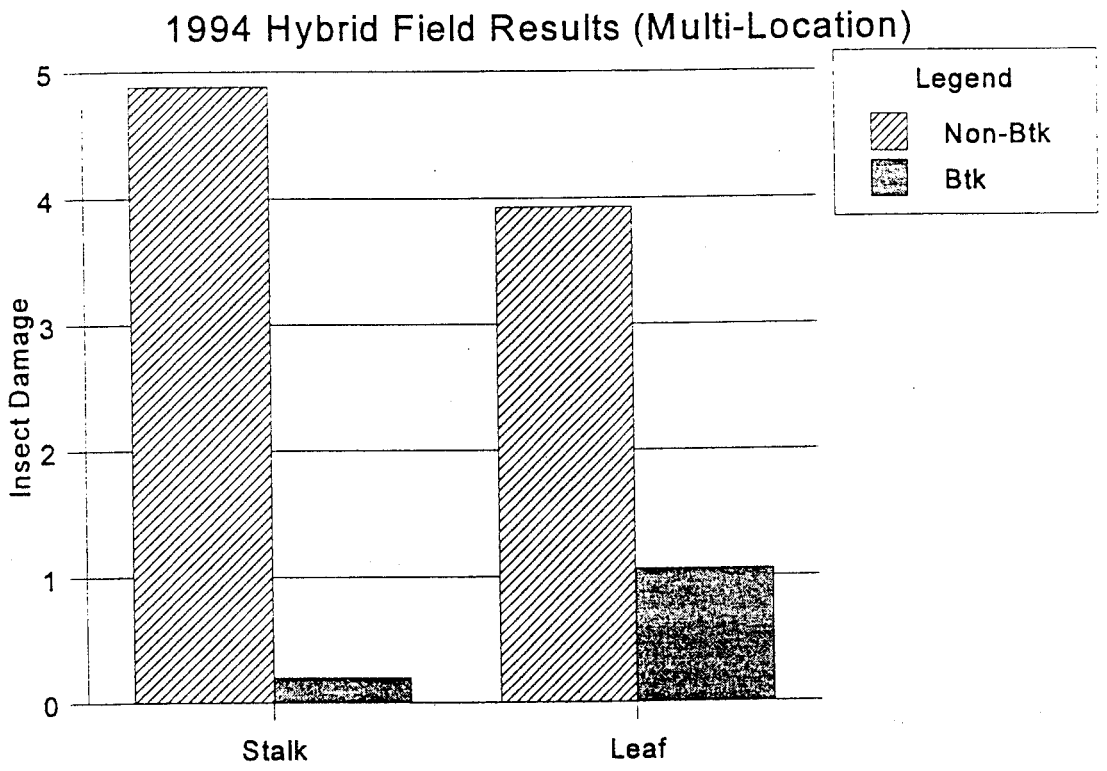
Results:

Figure E1 displays the summarized results across all trials and locations. Leaf feeding damage scores were approximately 3.9 on non-Btk hybrids and 1.1 on average Btk hybrids (1 on 1-9 scale represents no damage). Stalk damage (displayed in centimeters tunneled per plant) was approximately 4.9 cm in the average non-Btk control hybrid. The average Btk hybrid displayed only approximately 0.2 cm of tunneling per plant. In all cases, the difference between Btk hybrids and non-Btk hybrids were significant at a P-value of less than 0.01 based on ANOVA and LSD mean separation.

Conclusions:

These results confirmed the previous two years of field research indicating that these Btk hybrids provide excellent season-long control of ECB. Btk hybrids appear as normal and healthy with no visually apparent differences compared to non-Btk hybrids other than the lack of ECB damage.

Figure E1. European Corn Borer Damage



Trials were artificially Infested with first generation ECB and second generation ECB. Stalk damage expressed as centimeters tunneled/plant. Leaf damage expressed on 1-9 rating scale (Guthrie *et al*, 1960) where 1 = no damage or a few pinholes. Average plant damage ratings generated on 14 Btk hybrids and 6 non-Btk control hybrids, across 6 locations.

Study F: **Northrup King Co. Naturally Infested Btk Hybrid Observation - 1994 Field Trials**

Authors:
Jonathan B. Sagers and David W. Mies
Northrup King Co., Stanton, MN

Materials and Methods:

In 1994, 37 sites were planted with Btk hybrid corn. At each of these sites, forty 17-foot two-row plots of corn were planted with a primary purpose of assessing the natural incidence of European corn borer. A second goal was to observe any other naturally occurring pests of corn on these sites. Each of these trials contained at least six Btk hybrids and six closely related non-Btk hybrids, plus representative Northrup King and competitor commercial hybrids adapted to those growing areas. These trials were broken into two maturity groups; hybrids adapted for the 110-115 day maturity growing regions of Iowa, Illinois, and Nebraska and those adapted to 105 day or less maturity found mostly in Minnesota, Wisconsin, Michigan, and the Dakotas.

At each of these sites, just prior to harvesting grain, ten plants per hybrid of one or two non-Btk hybrids were dissected and the stalk damage by ECB was estimated. The hybrids that were split were relatively susceptible to ECB damage, and served as an indicator of overall ECB pressure at that site. If the average damage per plant was equal to two inches (5.08 cm), ten plants of each of the rows was dissected and the individual plant damage ratings were recorded. If damage was less than two inches per plant on average, no more plant dissections occurred at that site.

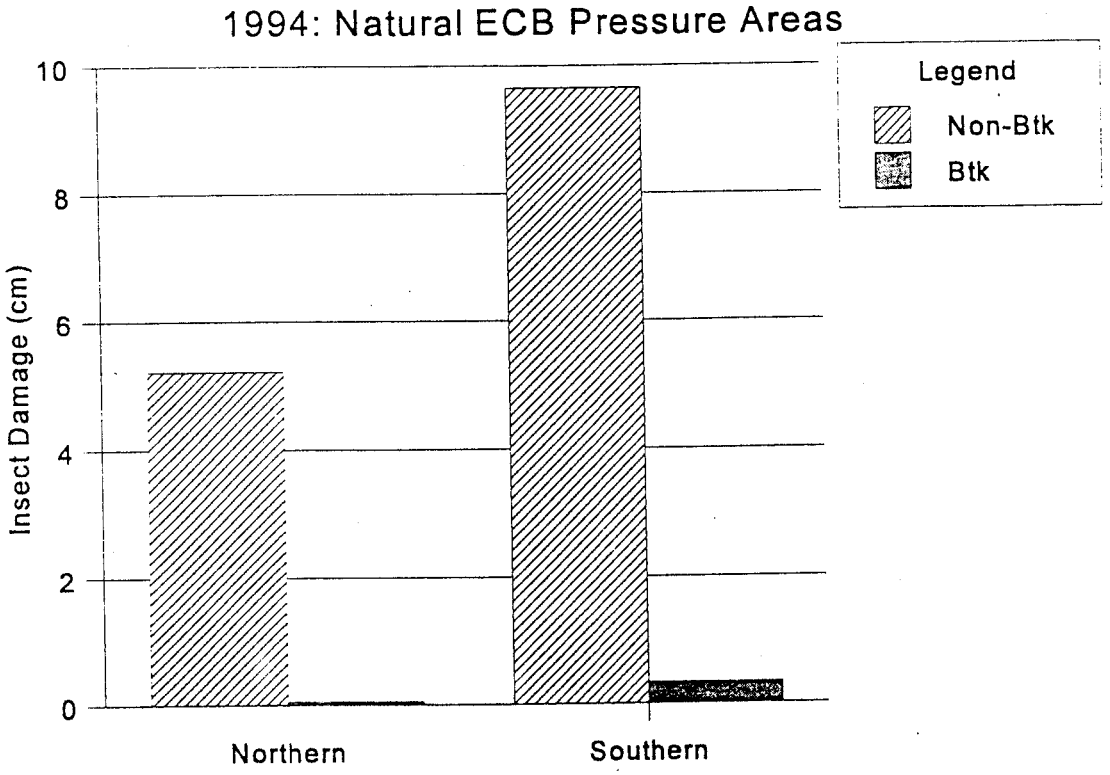
Results:

Figure F1 depicts the results of these multi-location trials. The figure distinguishes between northern adapted hybrids and southern adapted hybrid trial sites. Only the sites that had significant damage [two inches (5.08 cm) or more per average non-Btk hybrid] were included. Of sites planted with early maturity hybrids, northern Iowa locations of Hampton, Webster City, and Ogden were included. Sites planted with later maturity hybrids included Phillips, NE, Shennandoah, IA, and Hildago, IL. The figure shows that stalk damage for Northern adapted hybrids was just over 5.2 cm per plant in non-Btk hybrids and only approximately 0.15 cm per Btk hybrid plant. Plants at the southern sites sustained approximately 9.7 cm per plant in non-Btk hybrids compared to approximately 0.3 cm per plant in Btk hybrids.

Conclusions:

Btk hybrids evaluated in 1994 all provided excellent control of naturally occurring European corn borers. These results correlate well with what has been observed in artificially infested ECB trials conducted over the past three years. It is expected that these Btk hybrids will sustain very little damage by ECB anywhere the hybrids are grown.

Fig. F1: European Corn Borer Stalk Damage



Trials were under natural ECB pressure. Northern trials consisted of three northern Iowa locations. Southern trials consisted of three locations in southern Iowa and Nebraska. Stalk damage expressed as centimeters tunneled/plant. LSD $_{(a=0.05)}$: Northern hybrids=1.8cm; Southern hybrids=5.6cm.

Study G: Determination That AMPr Gene Not Present in Bt11 Transgenic Corn Line

Authors:
Irvin J. Mettler and John A. Hanten
Northrup King Co., Stanton, MN

The ampicillin resistance (AMPr) gene is required for molecular manipulations in the laboratory host *E. coli* but is not required for transformation in the plant. Without a plant promoter, the AMPr gene would not be expressed in a transformed plant. As an additional measure, the plasmid vector pZO1502 was designed to facilitate the production of transgenic plants that would not contain the gene for resistance to the antibiotic ampicillin. Before using the plasmid DNA for corn transformation it was digested with the restriction enzyme *Not* I. These unique restriction sites were incorporated around the AMPr gene sequence. *Not* I digestion effectively separated the AMPr gene from the Btk and PAT genes. Subsequent transformation and selection of transgenic events enabled us to identify the Bt11 corn line.

Transgenic seeds (event Bt11 backcrossed into inbred line 2044) were sown in the greenhouse and sprayed with Ignite^{R} herbicide at the four leaf stage. Resistant plants and control, untransformed, 2044 inbred plants were then used for DNA extraction and Southern blot analysis (T. Maniatis. E.F. Fritsch, and J. Sambrook, 1982. Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory). Probing these blots showed that no AMPr gene sequences were present in the Bt11 transgenic line. The complete lack of AMPr gene sequences was confirmed by additional Southern analyses and by PCR tests with specific AMPr gene primers (data not shown).

Table G1. Data summary from Southern blots shown in Figure G1. Five µg of genomic DNA was loaded per lane and run on a 1% agarose gel. The DNA probe fragment was isolated from the original plasmid vector pZO1502: AMPr = *Not* I fragment.

Blot #	Restriction enzymes	Probe	Predicted - Observed - # Fragments		
1	*Not* I	AMPr	1.1 kb	Nb*	0
2	*Hin*d III	AMPr	>1 kb	Nb	0
3	*Eco*R I	AMPr	>1 kb	Nb	0
4	*Pst* I	AMPr	>1kb	Nb	0

*Nb: No band observed.

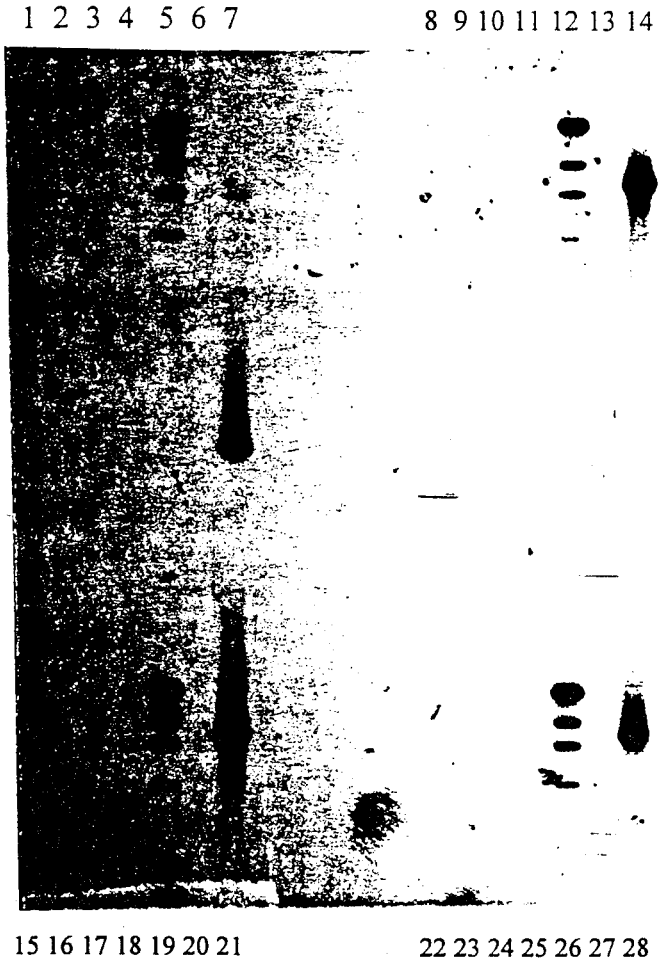


Figure G1. Genomic Southern analysis of Bt11 corn for AMPr gene sequences. Five µg of genomic DNA was loaded per lane, run on a 1% agarose gel, and blotted onto a nitrocellulose filter. The AMPr gene DNA probe fragment was isolated from the original plasmid vector pZO1502: AMPr = *Not* I fragment. DNA in lanes 1, 2, 8, 9, 15, 16, 22, and 23 are from two control, untransformed plants; lanes 3, 4, 10, 11, 17, 18, 24, and 25 are from two Bt11 plants; lanes 5, 12, 19, and 26 were loaded with the lambda (digested with *Hin*d III) DNA size standard; lanes 7, 14, 21, and 28 contain the pZO1502 DNA positive control; and lanes 6, 13, 20, and 27 are blank. Lanes 1 to 7 were digested with *Not* I; 8 to 14 with *Hin*d III; 15 to 21 with *Eco*R I; and 22 to 28 with *Pst* I.

Study H: Classical Determination of Inheritance and Gene Stability

Authors:
Irvin J. Mettler, Andrew S. Wang, Jonathan B. Sagers, and Rose A. Evans
Northrup King Co., Stanton, MN

To determine whether the Btk gene was inserted in more than one locus in the genome of the Bt11 line, the segregation of the Btk gene and the selectable herbicide resistance gene was followed in multiple generations. Eight F1 plants were identified as containing the Btk and PAT genes. They were selfed to produce an S1 population. This S1 population was screened for resistance to the European corn borer (ECB) and Ignite^{R} herbicide. All plants were either resistant to ECB and Ignite or susceptible to both. The segregation ratios are given in Table H1 and are consistent with an expected ratio of 3:1 for a single dominant locus.

The S1 plants were again selfed. Seed was collected from 96 of the ECB and PAT resistant plants and planted in the Northrup King Co., Stanton, MN research field (~30 plants per row) on May 20, 1994 for analysis. 2320 plants were obtained and infested with ECB three times (on 6/23, 6/27, and 6/30) by NK Entomology department. Leaf tips were "painted" with 1% Ignite^{R} on 7/14. This limited application of herbicide can detect Ignite resistance without severely damaging susceptible plants. 134 plants of HA and HP inbreds were used as negative controls. They were also infested with ECB and painted with 1% Ignite^{R}. Data were collected on 7/25/94. Segregation ratios identifying homozygous and heterozygous lines (S2) were tabulated separately for final analysis of segregation ratios.

Conclusions: The data summarized in Table H1. clearly show:
1. The Btk gene is inherited as a single locus in the Bt11 line (fits 3:1 ratio well in S1 and S2-Hetero population).
2. The PAT gene is also inherited as a single locus in the Bt11 line.
3. The Btk and PAT genes are closely linked (of 2320 S2 plants, no segregation of the two genes was found).

Table H1. Segregation of the Btk and PAT genes in S1 and S2 populations of Bt11. Ratios represent the number of plants resistant to ECB and Ignite^R to the number sensitive. Each S2-hetero population and the total S1 and S2 populations were tested (Chi-square) for the expected 3:1 segregation ratio. The individual X^2 and p values are given for the S2 population.

Initial F1 Parental plant	S1 Population	S2 - Homo Population	S2- Hetero Population	X^2 p
1	25:10	101:0 (4)*	57:25 (3)*	1.33 .25
2	25:8	18:0 (1)	57:25 (7)	0.03 .80
3	17:7	54:0 (2)	131:45 (4)	0.00 1.00
4	12:10	49:0 (2)	81:27 (1)	2.71 .10
5	24:11	163:0 (6)	24:3 (8)	0.50 .50
6	29:7	331:0 (13)	187:56 (9)	0.50 .50
7	23:11	137:0 (7)	173:61 (10)	0.16 .65
8	26:8	164:0 (8)	164:52 (11)	0.14 .70
Total	181:72 (p=0.2)	1017:0 (43)	983:320 (53)	0.14 .70
HA and HP Negative controls			0:134	

*() Number of S1 plants contributing seed for S2 progeny test.

Study I: **Determination of Insertion Site of the Transgenic Event Bt11**

Authors:
Marlin D. Edwards, Douglas Mead, and Marlin D. Edwards
Northrup King Co., Stanton, MN

The insertion site for the Bt11 event was mapped to the long arm of chromosome 8. The mapping process was as follows:

The original genetic stock into which the Btk sequence was transformed is coded BG for the purposes of this petition. The T_0 plant was used as a pollen donor for crosses to two, elite, inbred lines for which Btk-conversion was sought. Multiple backcrosses were conducted with selection of individuals that contained the insertion sequence but were, otherwise, as much like the elite recurrent parents as possible.

Four or more backcrosses and selfing to homozygosity were used in the conversion process. "Finished" conversion stocks were evaluated with a series of 50 to 60 RFLP probes selected to be well distributed throughout the genome. Genotypes of the Btk conversions were compared to those of their recurrent parent "isolines". These were generally identical, or nearly identical for all genetic markers, except for three probes on a small segment of the long arm of chromosome 8. All conversion stocks differ from the genotype of the transformed stock, HE89, for this segment, thus differing from their recurrent parents. There were no other genomic regions with consistent differences between Btk-conversions and their recurrent parents. These three probes exist within 10 centiMorgans (cM) of one another at the approximate position of the public probe UMC30a, which has been placed at map position 117 in the 1995 map of RFLP probe positions distributed by the University of Missouri at Columbia.

A series of 95 backcross 4 progeny were characterized for numerous probes in the region of chromosome 8 identified above. The size of the "donor" DNA segment varied among these progeny. However, five of the progeny failed to contain the donor alleles at the flanking markers: Z1B3 and UMC150a, despite presence of the inserted Btk sequence. These two probes are approximately 15 cM apart on chromosome 8. Thus, we are certain that the insertion site is within approximately 15 cM of chromosome 8, near position 117, and in the interval flanked by the two markers: Z1B3 and UMC150a.

Study J: **Southern Analysis of Transgenic Event Bt11 - Insert Copy Number**

Authors:
Irvin J. Mettler and John A. Hanten
Northrup King Co., Stanton, MN

Transgenic seeds (event Bt11 backcrossed into inbred line 2044) were sown in the greenhouse and sprayed with Ignite^{R} herbicide at the four leaf stage. Resistant plants and control, untransformed, 2044 inbred plants were then used for DNA extraction and Southern blot analysis (T. Maniatis, E.F. Fritsch, and J. Sambrook, 1982. Molecular Cloning A Laboratory Manual. Cold Spring Harbor Laboratory). The genomic DNA samples were digested with the following restriction enzymes and probed with labeled DNA for the Btk and PAT gene sequences. The first enzyme combination utilized 2 restriction sites present on the plasmid DNA. The next two enzymes had only one known location and would be expected to cut the genomic DNA at a distant site in the plant's DNA sequence. The actual size of any observed fragment depends on the insertion event. The number of bands can be used to estimate insertion copy number - each gene copy would produce a unique band on the Southern blot.

The results shown in Figures J1 and J2 are summarized in Table J1. These data show that the Bt11 transgenic lines are derived from a single insertion event containing one gene copy of the Bt and PAT gene sequences.

Table J1. Data summary from Southern Blots shown in Figures J1 and J2.
Five µg of genomic DNA was loaded per lane and run on a 1% agarose gel. The DNA probe fragments were isolated from the original plasmid vector pZO1502: Btk = *Sal* I & *Sac* I fragment and PAT = *Sal* I fragment.

Blot #	Restriction enzymes	Probe	Predicted - Observed - # Fragments		
1	*Sal* I and *Sac* I	Btk	1.3 kb	1.3 kb	1
2	*Hin*d III	Btk	>3 kb	~30 kb	1
3	*Eco*R I	Btk	>5 kb	~25 kb	1
4	*Pst* I and *Hin*d III	PAT	1.5 kb	1.5 kb	1
5	*Hin*d III	PAT	>2 kb	~30 kb	1
6	*Eco*R I	PAT	>5 kb	~25 kb	1

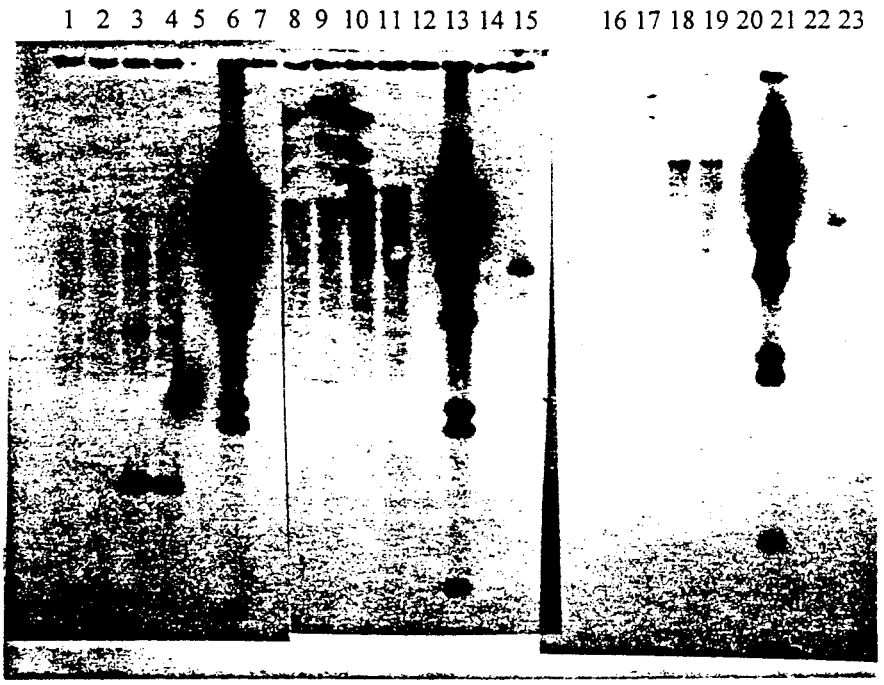


Figure J1. Genomic Southern analysis of Bt11 corn for Btk gene sequences.
Five μg of genomic DNA was loaded per lane and run on a 1% agarose gel. The Btk gene DNA probe fragment was isolated from the original plasmid vector pZO1502:Btk = *Sal* I and *Sac* I fragment. DNA in lanes 1, 2, 8, 9, 16, and 17 are from two control, untransformed, plants; lanes 3, 4, 10, 11, 18, and 19 are from two Bt11 plants; lanes 6, 13, and 21 were loaded with the lambda (digested with *Hin*d III) DNA size standard; lanes 15, and 23 contain the pZO1502 DNA positive control; lanes 5, 7, 12, 14, 20, and 22 are blank. Lanes 1 to 7 were digested with *Sal* I and *Sac* I; 8 to 15 with *Hin*d III; and 16 to 23 with *Eco*R I.

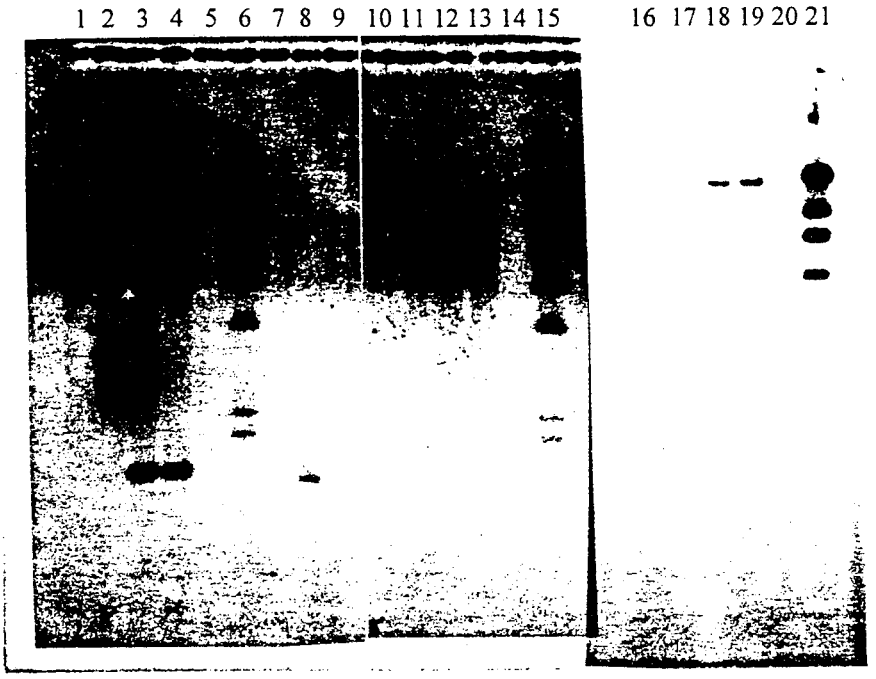


Figure J2. Genomic Southern analysis of Bt11 corn for PAT gene sequences.
Five μg of genomic DNA was loaded per lane and run on a 1% agarose gel. The PAT gene DNA probe fragment was isolated from the original plasmid vector pZO1502: PAT = *Sal* I fragment. DNA in lanes 1, 2, 10, 11, 16, and 17 are from two control, untransformed, plants; lanes 3, 4, 12, 13, 18, and 19 are from two Bt11 plants; lanes 6, 15, and 21 were loaded with the lambda (digested with *Hin*d III) DNA size standard; lane 8 contains the pZO1502 DNA positive control; lanes 5, 7, 9, 14, and 20 are blank. Lanes 1 to 9 were digested with *Pst* I and *Hin*d III; 10 to 15 with *Hin*d III; and 16 to 21 with *Eco*R I.

Study K: Level of Btk Protein in Transgenic Plant Tissues During Plant Life Cycle

Authors:
Irvin J. Mettler and David Farrar
Northrup King Co., Stanton, MN

To estimate the level of Btk protein produced by transgenic plants, various plant tissues were analyzed for Btk protein during the growth of the plants. Bt11 transgenic plants were grown in the greenhouse. Five (minimum) replicate plants were sampled for each data point. Plant tissue extracts were produced and assayed for Btk protein by ELISA as described in, Determination of Levels of Plant-Produced *Bacillus thuringiensis kurstaki* HD-1 Protein in Transgenic Maize, EPA MRID #43397201, and Appendix G. All data were expressed as a specific concentration: ng Btk protein per mg of plant protein extracted.

As shown in Table K1, highest Btk protein concentrations were found in the leaf tissue. Generally, higher levels were detected at the younger stages of tissue development. Btk protein was detected in all plant tissues sampled. The level of Btk protein decreased as the plant reached full maturity and the tissues became senescent (day 119).

Table K1. Specific concentration of Btk protein in Bt11 transgenic corn tissues during the life cycle.
Plants were grown in the greenhouse and five replicate plants were extracted for each destructive sample time. Data are expressed as ng Btk protein/mg plant protein (Standard Error of Mean) and are not corrected for actual extraction efficiency. Samples were removed from plants (or the entire plant sacrificed) at the indicated days post-planting.

Tissue	5	10	15	20	25	30	37	59	84	119
Cotyledon	20.5 (0.4)	36 (1.7)								
Roots	22.1 (1.3)	11.7 (0.8)					37 (7)	12 (3.4)	18.2 (4)	2.2 (1.2)
2nd leaf		106 (4.7)	27.9 (3)	22.4 (0.9)	125 (5)	38 (1.3)	55.6 (4)			
5th leaf				45.7 (2)	168 (5)	34 (1.3)	54 (3.3)	16.7 (1.2)		
10th leaf							102 (6)	30 (1.5)	9.4 (1)	

Tissue	5	10	15	20	25	30	37	59	84	119
15th leaf								37.9 (2.2)	10.2 (1.1)	
Stalk epidermis							36 (3.3)	10.4 (2.6)	12.6 (3.4)	9.0 (2.2)
Stalk pith							27 (4)	19.2 (3.1)	18.0 (4.8)	8.8 (2.0)
Tassel								8.0 (1.4)	8.8 (2.0)	6.8 (4.2)
Pollen								1.25 (.75)		
Silk								2.4 (0.6)	6.6 (1.8)	5.2 (3.8)
Ear Shank								13.6 (2.3)	27.2 (8.8)	5.2 (1.4)
Husk								24.8 (2.9)	15.4 (5.3)	2.6 (2.6)
Cob								13.0 (3.0)	26.6 (6.4)	16.2 (3.3)
Brace root								3.2 (1.2)	7.0 (2.1)	4.8 (2.1)
Kernel									8.2 (2.5)	0.4 (0.4)

Study L: Quantity of Btk Protein Present in an Acre of Transgenic Corn (Bt11)

Authors:
Marlin D. Edwards, Irvin J. Mettler, and John A. Hanten
Northrup King Co., Stanton, MN

Estimates of the quantity of Btk protein present in an acre of transgenic corn were determined using available data on silage corn biomass production, dry matter partitioning in the corn plant, moisture status of corn plants, and measured Btk protein concentrations in fresh corn tissue from transformed plants.

Average biomass production was based upon replicated variety trials of maize harvested for determination of silage yield over multiple years (proprietary data) and from the document NCH-49 of the "National Corn Handbook", published by the Iowa State University cooperative Extension Service. A value of 12.6 tons/acre (dry matter basis) was chosen as representative of optimal corn belt yields of silage plus root biomass under conventional agricultural practices (data calculated for R6 Stage of corn development - physiological maturity, ~ 55 to 65 days after silking). The fraction of this biomass that is attributable to various plant parts was determined using data from Modern Corn Production, S.R. Aldrich, et al., publ. by A & L Publications, Champaign, IL and from How a Corn Plant Develops, Special Report 48, Iowa State University Cooperative Extension Service, January 1986.

Extractible Btk protein as a fraction of total tissue weight (fresh weight basis) was determined for plant tissues expressing Bt11 by Northrup King Co. scientists. The measured concentration of Btk protein in grain and leaves was 1.4 and 3.26 µg Bt protein/g fresh weight (Determination of Levels of Plant-Produced *Bacillus thuringiensis kurstaki* HD-1 Protein in Transgenic Maize, EPA MRID #43397201, and Appendix G). The specific concentration of Btk protein in stalk, ear shank, tassel, cob, roots, and silks (Table L1) was averaged and corrected for protein level relative to leaf material to provide the estimated concentration of 0.14 µg Btk protein/g fresh weight of stalk tissues. The recovery efficiency of Btk protein was estimated from samples of leaf and grain (Determination of Levels of Plant-Produced *Bacillus thuringiensis kurstaki* HD-1 Protein in Transgenic Maize, MRID #43397201, and Appendix G) and used to estimate the total amount of Btk protein present in the plant.

Our estimate of the total Btk protein produced per acre of corn is 0.57 pounds. This is based on a total biomass of 89,300 lbs. of fresh tissue per acre. Nearly 90% of the total Btk protein in the plant is located in the leaf tissue (Table L1).

Table L1. Estimated amount of Btk protein present in an acre of Bt11 corn.
These calculations are based on silage corn biomass production, dry matter partitioning in the corn plant, moisture status of corn plants, and measured concentrations of Btk protein in fresh corn tissue from transformed plants. Data calculated for corn plants at physiological maturity (R6 stage, ~ 55 to 65 days after silking).

Tissue	Dry Weight Lbs/Acre	Fresh Weight Lbs/Acre	Btk Conc. µg/g FW	Extraction Efficiency	Total Btk Lbs/Acre
Leaves[1]	4,900	32,700	3.26	0.21	0.508
Stalk[2]	11,600	46,400	0.14	0.30	0.022
Grain	8,700	10,200	1.40	0.38	0.038
Total	25,200	89,300			0.57

[1] Leaves include: leaf, leaf shank, and husk.
[2] Stalk includes: stalk, ear shank, tassel, cob, roots, and silk.

Study M: **Fate of Btk Protein in Transgenic Plant Material and Soil**

Authors:
Irvin J. Mettler and Barbara J. Bolan
Northrup King Co., Stanton, MN

It has generally been assumed that the Btk protein produced in transgenic plants will have the same environmental fate as other plant proteins. As the plant dies and its tissues are degraded in the soil, the Btk protein would simply be degraded along with the other plant proteins. As a confirmation of this assumption, we have directly measured the level of Btk protein in leaves and stalks of transgenic corn plants subjected to a simulated degradation cycle. Btk protein was also added directly to soil samples as a further test for Btk protein persistence or degradation in the soil.

Materials and Methods:

Stalk and leaf material were harvested from mature field-grown corn plants derived from transgenic line Bt11 (backcrossed into line HAF031 and grown in Hawaii for seedstock). Non-transformed 2044 inbred tissue was used as a control. Harvested material was fresh frozen and shipped to the Northrup King Co. research facility in Stanton, MN. Stalk tissues were split in half. Leaf and stalk samples were weighed and added to 60 g field soil (a fine silt loam purchased from Farmers Union Coop Oil, Randolph, MN) in a plastic Petri dish. Tissue samples were either placed on the soil surface or buried in the soil. Soil samples without plant tissues were spiked with purified, Btk protein (Bt103 standard [see Volume 6 to this submission], 1 mg/60 g soil). The soil and tissue samples were maintained in a greenhouse (16-hr day, 2066 foot candles, 80° F, and 8-hr night, 70° F) and watered daily to maintain moisture (not saturated). Replicate samples were prepared to provide material for analysis at 0, 1, 2, and 3 weeks.

At the one week intervals, tissue and soil samples were collected and processed for analysis of Btk protein, and total protein. Soil samples from 0 and 3 week treatments were tested for insecticidal activity against European corn borer larvae. Tissue samples were ground in liquid nitrogen, mixed with ~6 volumes of extraction buffer (50 mM bis-Tris propane, pH 7.5, 5 mM ethylene-diaminotetraacetic acid, 5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride), incubated at 4° C for 30 to 60 min, centrifuged at 11,000 x g for 20 min., and the levels of Btk and total protein determined by ELISA (described in: Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein, EPA MRID #43397202, and Appendix H) and Bio-Rad Protein assay. The calculated weight of tissue samples was adjusted to represent an equivalent proportion of initial material. The actual weight of material collected was typically 50% less than the starting weight. Soil samples were mixed with ~6 volumes of extraction buffer and the level of Btk and total protein determined as for tissue samples.

For Western analysis of soil treatments, samples were extracted with boiling SDS buffer (50 mg soil/50 µl or 10 mg soil 100 µl of 4% sodium dodecyl sulfate (SDS), 125 mM Tris pH 6.8, 20%

glycerol, and 50 mM dithiothreitol, DTT) for 20 min. and then processed by standard methods for Western blotting, (see previously submitted study, Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein, EPA MRID #43397202).

Insecticidal activity assays were conducted with the samples by incorporating 10% of the ground plant material or the soil samples into the diet medium for ECB growth as previously described (in: Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein, EPA MRID #43397202). Thirty-two neonate larvae were used for each treatment tested. Insect mortality and average weights were determined after 8 days.

Results and Discussion:

Btk and total protein levels in plant samples.

As expected, the best data were obtained from the initial time point. Later samples had very low levels of detectible Btk protein and were also more difficult to collect due to tissue degradation and contamination with soil. As shown in Table M1 the level of detectible Btk and total plant protein dropped rapidly. After one week, ~ 1 and 10% of the original Btk protein in the leaf and stalk tissues, respectively, was detected by ELISA. After three weeks, Btk protein was still detected in the stalk tissue but the level in transgenic leaves was similar to the background levels seen in control leaf tissues. This degradative trend was also seen for the level of total protein in the tissue samples. These data indicate that the degradation of Btk protein in plant tissue samples was even more rapid than the general loss of plant protein. This is particularly apparent for the leaf samples where the average specific concentration of Btk protein decreases from 80.4 in the initial leaf sample, to 5.4 and 1.3 (ng Btk/mg protein) after 1 and 2 weeks incubation.

Btk and total protein levels in soil samples.

Quantitative extraction of Btk protein directly from the spiked soil was not possible. Apparently, the Btk protein bound to the soil particles and was not released by the standard extraction buffers.

A Western blot of the spiked soils showed that Btk protein could be detected in the initial spiked soil (when extracted with the boiling SDS buffer) sample but was no longer detectible after one week.

Bio-Rad protein assays showed low levels of reactive material could be extracted from the soil (mean: 0.08 mg /g soil ± 0.004 SE). There was no correlation between the level of protein detected and the sample treatment or incubation time.

Insect activity assays with tissue and soil samples.

The plant tissue and soil samples collected at initial time zero and after three weeks were tested for insecticidal activity against ECB larvae (Table M2). Significant mortality was only observed with the insects fed the purified Bt103 standard. Significant growth inhibition was seen for ECB larvae fed extracts of transgenic leaves at time zero. After the three-week incubation period, extracts of leaf material no longer inhibited the growth of the target insects. Unexpected growth inhibition was obtained with control, non-transformed, leaf tissues extracted at time zero and after the three-week greenhouse incubation. Since this was field-grown material, the control plants may have contained residues from chemical pesticides that would have been applied to these seedstock nurseries.

Table M1. Degradation of Btk protein in transgenic leaf and stalk tissues.

Transgenic tissue samples were incubated with 60 g soil under greenhouse conditions. After 0, 1, 2 and 3 weeks, the tissue was harvested, weighed, ground, and analyzed for presence of Btk protein and total protein. Data expressed as: ng Btk protein/mg total protein; µg Btk protein/g tissue (equivalent weight); and mg total protein/g tissue (equivalent weight). Each data point represents the mean of four replicate samples.

Tissue, Treatment	Btk/ prot	Btk/ g tis	pro/ g tis	Btk/ prot	Btk/ g tis	pro/ g tis	Btk/ prot	Btk/ g tis	pro/ g tis	Btk/ prot	Btk/ g tis	pro/ g tis
	0 week sample			1 week sample			2 week sample			3 week sample		
Stalk above soil	15.3	0.03	1.69	9.03	.003	0.35	7.73	.002	0.22	9.6	.001	0.13
Stalk below soil				8.2	.002	0.23	12.6	.002	0.48	11.4	.003	0.29
Leaf above soil	80.4	1.10	13.4	3.89	.014	3.17	1.69	.003	2.02	<0	<0	1.19
Leaf below soil				6.90	.009	1.09	0.91	.003	1.30	5.65	.002	0.53
Ctrl. Stalk combined	0.56	.004	6.86	1.39	.0006	1.26	0.75	.0006	0.62	2.73	.0009	0.45
Ctrl. Leaf combined	0.40	0.01	30.1	0.20	.0005	3.30	0.30	.001	2.36	1.95	.002	0.84

Table M2. Growth of European Corn Borer on diet incorporating plant tissues and soil. Data represent combined results for tissues above and below soil. Each separate treatment tested with 32 neonate larvae.

Treatment	Week 0 samples		Week 3 samples	
	Ave. wt (mg)	Mortality %	Ave. wt (mg)	Mortality %
Bt103 25 ppm	0.2	75.0		
Bt103 12.5 ppm	.02	56.3		
Bt103 6.25 ppm	.04	46.9		
Bt103 0.25 ppm	6.3	9.4		
Control 0 ppm	6.5	4.7		
Bt11 leaf	.6	13.3	4.6	2.6
Bt11 stalk	5.1	12.5	6.8	8.3
Control leaf	2.2	9.4	1.4	9.4
Control stalk	9.7	3.1	7.3	14.6
Soil-Bt11 leaf			7.1	6.7
Soil-Bt11 stalk			6.5	9.9
Soil-Control leaf			6.0	14.1
Soil-Control stlk			7.2	7.9
Control soil	4.4	6.3		
Soil+Bt103 (1.7ppm)	3.6	21.9	5.7	15.6

Study N: **Detection of Enzymatic Activity of Phosphinothricin Acetyl Transferase (PAT) in Bt11 Corn**

Authors:
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Northrup King Co., Stanton, MN

A radioactive enzymatic assay was used to determine the presence of the PAT enzyme in transgenic corn lines (including the Bt11 line). The production of the PAT enzyme has typically been verified by the ability of the transgenic plants to escape injury when sprayed or "painted" with the herbicide. The herbicide is rendered non-toxic by the enzymatic acetylation.

We have also developed a simple *in vitro* enzymatic assay to verify the presence of the PAT enzyme activity in plant tissue samples. This assay utilizes a radioactive substrate (^{14}C-acetyl CoA) to detect the acetylated product.

Assay Protocol

Fresh tissue sample (30-50 mg) is ground (on ice) in ~5 volumes of extraction buffer (100 mM Tris-HCl. 0.3 mg/ml dithiothreitol, and 0.3 mg/ml bovine serum albumin (BSA fraction V). The homogenate was centrifuged to clarify (12,000 x g for 5 min). Approximately 2 μl of extract was added to the reaction mixture containing the extraction buffer plus 125 μM acetyl CoA and 250 μM phosphinothricin (control reactions deleted the ppt substrate). The enzymatic reaction was allowed to proceed for 1 hr at 37^0 C. The reaction mix was then spotted onto TLC silica gel plates (Baker Si250-PA (19C)). The plate was chromatographed for 2-3 hr with isopropanol:NH_4OH (3:2), air dried and vacuum dried in an oven at 80^0 C. The radioactive spots are then exposed to X-ray film for 1-4 days. The results of a typical assay is shown in Figure N1.

This procedure allows us to confirm the presence and activity of the PAT enzyme in transgenic plant samples (see Figure N1, following). Due to the lability of the PAT enzyme, the enzymatic assays are routinely conducted with fresh tissue and on freshly isolated extracts.

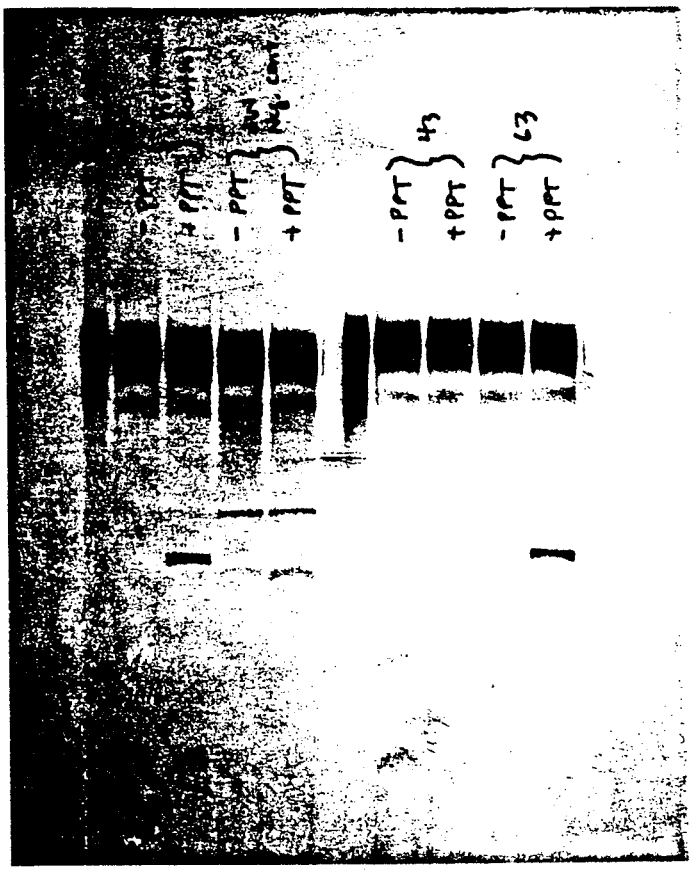

Figure N1. Autoradiograph of phosphinothricin acetyl transferase activity in transgenic plant and callus samples. Fresh tissue extracts were incubated with ^{14}C acetyl CoA with and without phosphinothricin substrate for 1 hr at 37^0 C. The reaction mixtures were spotted onto silica gel plates and the reaction products separated. After drying, the radioactive products were visulalized by exposure to X-ray film for 2 days. Lanes 1 and 2 from Bt transgenic corn leaf. Lanes 3 and 4 from control, untransformed leaf tissues. Lanes 5 through 8 demonstrate levels of activity obtained from two separate callus tissue samples selected for PAT resistance. Callus line 43 (lanes 5 and 6) shows very weak enzyme activity. Callus line 63 had significant levels of gene expression similar to Bt transgenic plant.

IV. Description of Differences in Genotype Between Regulated Article and Nonmodified Recipient Organism

Gene Donor Organisms:

Bacillus thuringiensis var. *kurstaki* strain HD-1

The Btk gene present in corn line Bt11 is a modified version of a gene originally isolated from *Bacillus thuringiensis* var. *kurstaki* (Btk). Btk is a spore forming soil microorganism which produces crystalline proteins. When Btk is ingested by certain lepidopteran insects, the proteins are solubilized in the alkaline conditions of the insect gut and proteolytically cleaved to form core fragments which are toxic to the insect. The core fragments specifically damage the cells of the midgut lining, affecting the osmotic balance. The cells swell and lyse, leading to eventual death of the insect. (Hofte and Whiteley, June 1989, *Microbiological Reviews*, pp 242-255).

Formulations of *Bacillus thuringiensis* have been used as biological pesticides for over three decades. Varieties have shown specificity for lepidopterans, dipterans and/or coleopterans. The protein encoded by the Btk HD-1 gene, CryIA(b), is specific to lepidopterans.

Streptomyces viridochromogenes

Streptomyces viridochromogenes is a gram-positive, sporulating, soil-inhabiting bacterium. It produces an enzyme, phosphinothricin acetyl transferase, which protects itself from a tripeptide, phosphinothricyl-alanyl-aline (Ptt), which it also produces, and which shows broad spectrum toxicity to plants. The gene which encodes the enzyme has been designated PAT.

An analogue of Ptt has been synthesized and is sold as the herbicide, glufosinate, under the trade names Basta (in Europe) and Finale (in the United States). It is being tested in the United States under the trade name Ignite, for use on resistant corn and soybeans. Glufosinate inhibits the glutamine synthetase of plants, resulting in an accumulation of ammonia in plant tissue, which leads to death of the plant.

The expression of the PAT gene, modified as described above for use in corn, protects the corn from the herbicide glufosinate. The encoded enzyme catalyzes the acetylation of phosphinothricin which prevents the herbicide from inhibiting glutamine synthetase. Therefore, an application of Ignite^{R} enables the selection of individual plants expressing the PAT gene and thus carrying the linked Btk gene. Once the presence of the PAT enzyme is established as a homozygous trait in the corn, Ignite^{R} will no longer need to be used as a selection tool. The regulated article for which this petition is made will not be marketed as an herbicide resistant corn line, although it will carry a label identifying it as carrying a glufosinate resistance gene (see Appendix D).

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Additional information on *Streptomyces viridochromogenes* and the PAT enzyme is presented in Appendix F, Toxicity/Allergenicity Considerations: Phosphinothricin Acetyl Transferase.

Transformation System:

The initial parental transformation of the corn lines to be planted under this registration was accomplished through insertion of a plasmid, pZO1502, containing the Btk and PAT genes, into the genome of corn. The transformation was performed [

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.] The material was grown up and crossed and backcrossed to non-transformed Northrup King Co. elite inbreds. Descendants of the initial crossings have been successively backcrossed and test crossed in order to establish and then evaluate different corn lines carrying the Btk gene. The corn to be grown under this petition is descended from the initial transformation event, and given the designation Bt11.

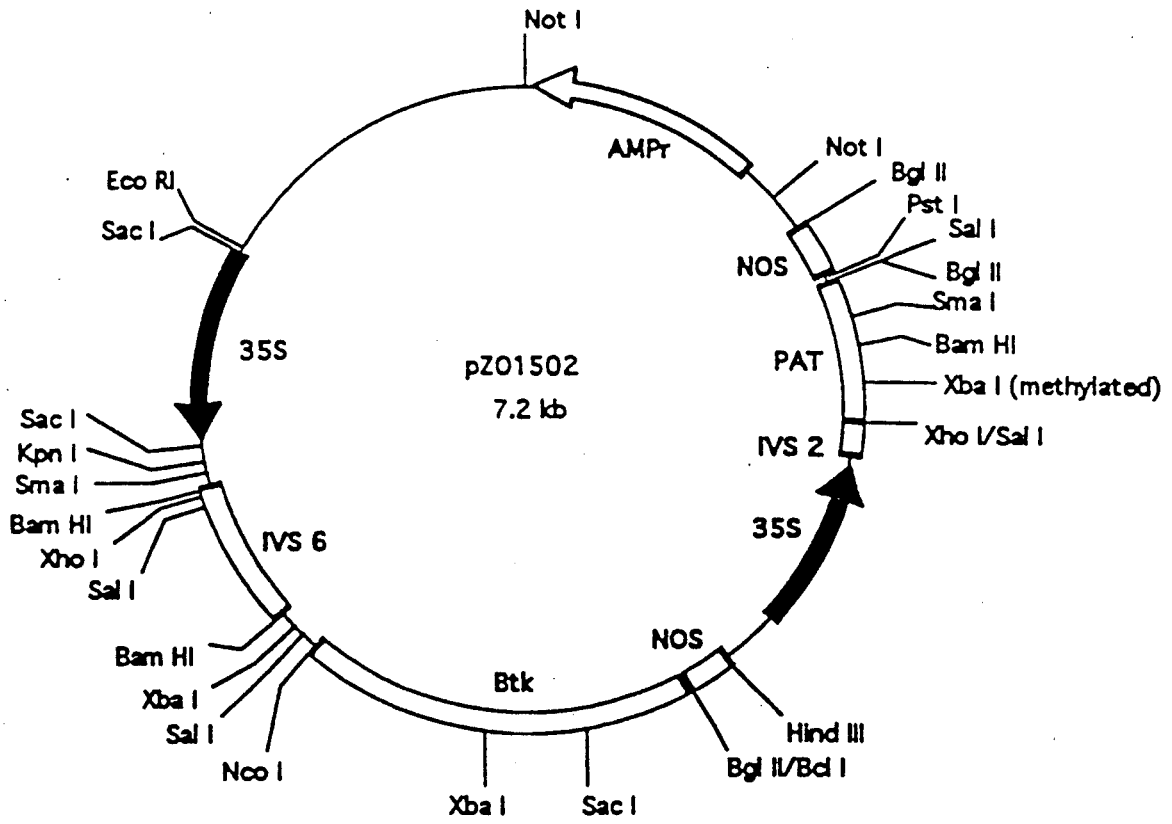
Plasmid Vectors:

pZO1502 Plasmid

The pZO1502 plasmid (see diagram on next page) consists of a 35S-1/intron/Btk HD-1/NOS cassette in the polylinker cloning site and a 35S-2/ intron/PAT/NOS cassette in the *Bgl* II site of plasmid pZO997. Both cassettes have the same direction of transcription with respect to the plasmid. pZO997 was constructed by converting the *Bsp* HI sites (at bp1526 and 2534) of pZO930 to *Not* I sites by fill-in followed by linker ligation. pZO930 was made by filling the *Eco* O 109I site of the base plasmid pUC18 (Yanisch-Perron, C., Vieira, J. and Messing, J., 1985, *Gene* 33:103-119) and ligation of *Bgl* II linkers.

The AMPr gene was used as a marker when the plasmid was being generated in *E. coli*. When under the control of a procaryotic (bacterial) promoter, the gene confers resistance to the antibiotic ampicillin. The gene is not expressed in plant cells, however; and Southern blot analyses have shown that descendants of the Bt11 line do not carry the AMPr gene. The evidence for this is presented in section III of this petition, in Study G: Determination that AMPr Gene Not Present in Bt11 Transgenic Corn Line. Thus, an antibiotic resistance gene is not present in Northrup King Co.'s Bt11 derived corn lines, as a result of this transformation event.

Diagram of plasmid pZO1502



Plasmid name: pZO1502
Plasmid size: 7.2 kb
Constructed by: P. Dietrich
Construction date: 10-12-1990
Comments/References: Vector: pZO1500 Eco + Hind III; Insert: pZO960 Eco + Hind III.

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Inserted Genetic Material and Its Products:

Coding Regions

Btk Gene:

The Btk gene, is a synthetic, modified version of the full length CryIA(b) gene of *Bacillus thuringiensis* var. *kurstaki* strain HD-1. The Btk gene was obtained from [ ] Modifications of the CryIA(b) gene include truncation and DNA sequence changes designed to enhance expression in plants, as described in [ ] Modifications did not result in any amino acid sequence changes. CBI DELETED

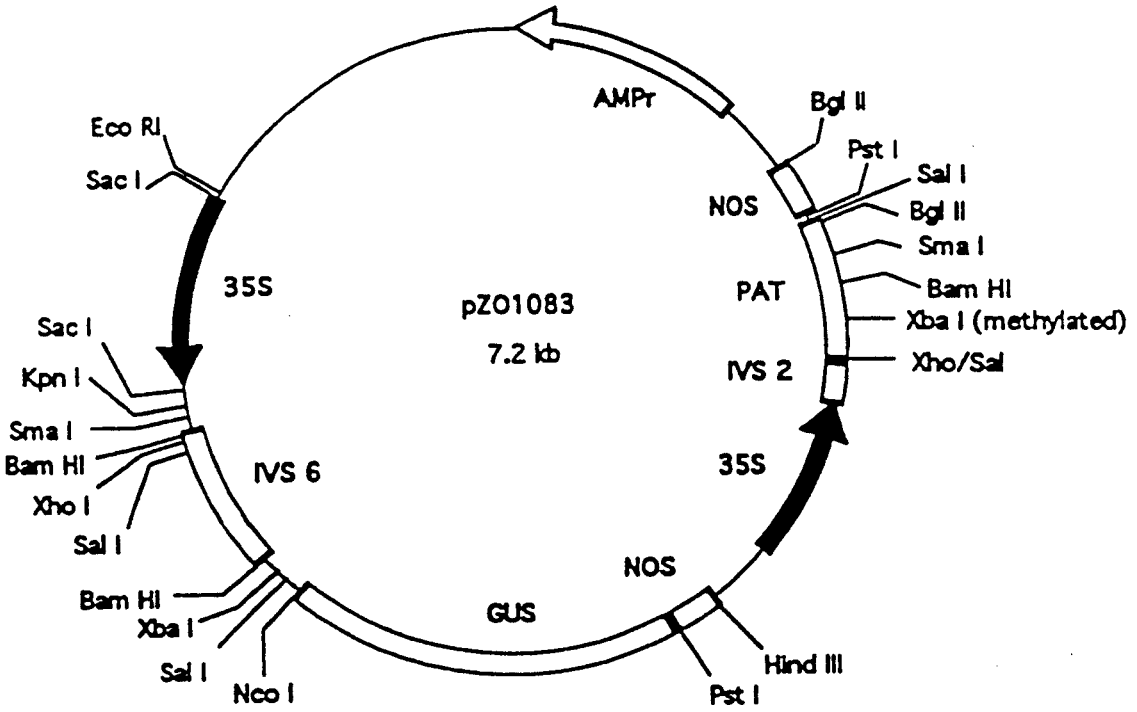
The product of the Btk gene is a truncated form of a delta-endotoxin protein from *Bacillus thuringiensis* var. *kurstaki*. The insecticidal activity of the Btk protein produced in Bt11 corn is equivalent to that of the microbially produced protein as demonstrated in the study Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein, EPA MRID #43397202, and Appendix H.

PAT Gene:

The phosphinothricin acetyl transferase (PAT) gene was cloned from the soil microorganism *Streptomyces viridochromogenes* strain Tu494 (Strauch, E., et al, 1988, *Gene* 63:65-74). The native sequence (Wohlleben, W., et al, 1988, *Gene* 70:25-37) was modified to optimize expression in plants. Modifications included changing the initiation codon from the native GTG to ATG and numerous codon changes designed to lower the GC content. The amino acid sequence was unaltered. The modified gene was obtained (as pOCA/Ac) from Dr. Peter Eckes, Massachusetts General Hospital, Boston, MA. The gene had been cloned as a *Sal* I fragment into pDH51 (Pietrzak, M. et al, 1986, *Nucleic Acids Res.* 14:5857), a pUC derived plasmid containing a 530bp version of the 35S promoter and a 220bp NOS terminator. The entire cassette had then been cloned as an *Eco* RI fragment (ca. 1.3kb) into the *Eco* RI site of pOCA18 (Olszewski, N.E., et al, 1988, *Nucleic Acids Res.* 16:10765-10782) to give pOCA/Ac. Upon receipt, the *Eco* RI fragment was subcloned from pOCA/Ac into pBluescript KS+ (from Stratagene, Inc. and derived from pUC19) to give pZO350. The gene was subsequently subcloned from pZO350 as a *Sal* I - *Pst* I fragment and combined with other fragments to form pZO1083 (see diagram on next page). The PAT cassette was then subcloned from pZO1083 into the *Bgl* II site of pZO997 (see pZO1502 Plasmid above).

The product of the PAT gene is the enzyme, phosphinothricin acetyl transferase. It provides resistance to the herbicide, glufonsinate, and is described in more detail earlier in this section, under **Gene Donor Organisms:** *Streptomyces viridochromogenes* and in Appendix F. Toxicity/Allergenicity Considerations: Phosphinothricin Acetyl Transferase.

Diagram of plasmid pZO1083



Plasmid name: pZO1083
Plasmid size: 7.2 kb
Constructed by: P. Dietrich
Construction date: 08-28-1989

Non-Coding Regions

35S Promoters:

The 35S promoters were derived from Cauliflower Mosaic Virus (CaMV), (Gardner, R.C., et al, 1981, *Nucleic Acids Research* 9:2871-2888 and Franck, A., et al, 1980, *Cell* 21:285-294). One originated from the CM1841 isolate of Cauliflower Mosaic Virus (CaMV), (Gardner, R.C., et al., 1981, *Nucleic Acids Research* 9:2871-2888). The other originated from the Cabb-S strain of CaMV (Franck, A., et al, 1980, *Cell* 21:285-294).

Introns:

The introns were derived from the maize alcohol dehydrogenase 1S gene (Freeling, M. and Bennett, D.C., 1985, *Ann. Rev. Genet.* 19:297-323). Use of these introns to enhance heterologous gene expression has previously been described (Mascarenhas, D., et al, 1990, *Plant Mol. Bio.* 15:913-920).

Terminators:

The NOS terminator consists of bp 423-678 of the nopaline synthetase gene of *Agrobacterium tumefaciens*, (Bevan, M., et al, 1983, *Nucleic Acids Res.* 11:369-385) plus added restriction sites.

Regulated Article:

The regulated article, for which this Petition for Non-Regulated Status is made, is all insect protected corn (*Zea mays* L.), derived from the initial transformation event designated by Northrup King Co. as Bt11, expressing the CryIA(b) gene from *Bacillus thuringiensis* var. *kurstaki.*

V. Description of the Phenotype of the Regulated Article

As part of the Btk corn backcrossing program at Northrup King Co., statistically rigorous studies were done on the performance characteristics of greatest commercial importance in a corn hybrid. These studies are presented in section III of this petition, as Study A: Analysis of Effects of Btk Conversion on Phenotypic and Yield Attributes of *Zea mays* L. Their results show that no differences were observed between hybrids derived using original elite lines and the selected Btk conversion lines for any of the traits measured: Yield, Moisture at Harvest, Root Lodging Rating, Ear Height, Plant Height or Heat Units to Silking or Pollen Shed.

Studies have also been performed to demonstrate the efficacy of the Btk conversion in providing protection from various Lepidopteran insect pests of corn. These studies are also presented in section III of this petition, as Study B: Southwestern Corn Borer, *Diatraea grandiosella*, and Fall Armyworm, *Spodoptera frugiperda*, versus Btk-Maize Hybrids; Study C: Northrup King Co. Btk-Maize Versus the Fall Armyworm, *Spodoptera frugiperda*; Study D: Northrup King Co. Btk-Maize Versus the Corn Earworm, *Helicoverpa zea*; Study E: Northrup King Co. Btk-Hybrid Maize Versus Artificially Infested European Corn Borer - 1994 Field Trials; and Study F: Northrup King Co. Naturally Infested Btk-Hybrid Observations - 1994 Field Trials. The results of these studies demonstrate that Northrup King Co.'s Btk hybrids show excellent potential for controlling European corn borer, southwestern corn borer, corn earworm, and fall armyworm.

In addition to the studies described above, Northrup King Co.'s staff of professional corn breeders, agronomists, and entomologists make observations on other characteristics to determine that the converted inbred lines and the resulting hybrids are equivalent to those of the original elite lines. The compiled and analyzed data from the various sites, together with a listing of the characteristics observed, are shown in Table V1, below, and demonstrate that no meaningful differences (except for those expected from the insect resistance provided by the Btk protein) are observed between the converted Btk lines and those from the original elite lines. The information in this table has been compiled from Field Trial Reports and Site Inspection Books, examples of which are shown in Appendices B and C, respectively.

Table V1. Comparison of Btk Converted and Original Elite Corn Lines for Various Characteristics.

Comments indicate the degree to which significant differences were observed for the seven characteristics listed.

Year: 1993

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
93-014-03	Henderson KY	none	none	none	none	none	none	none
	Morganfield KY	none	none	none	none	none	none	none
	Goerge IA	none	none	none	none	none	none	none
	Hampton IA	none	none	none	none	none	none	none
	Ottumwa IA	none	none	none	none	none	none	none
	Shennandoah IA	none	none	none	none	none	none	none
	Washington IA	none	none	none	none	none	none	none
	Webster City IA	none	none	none	none	none	none	none
	Hildalgo IL	none	none	none	none	none	none	none
	Mt. Vernon IL	none	none	none	none	none	none	none
	Peoria IL	none	none	none	none	none	none	none
	Rochelle IL	none	none	none	none	none	none	none
	St. Joseph IL	none	none	none	none	none	none	none
	Springfield IL	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	Hayfield MN	none	none	none	none	none	none	none
	Kenyon MN	none	none	none	none	none	none	none
	Stanton MN	none	none	none	none	none	none	none
	Fairbury NE	none	none	none	none	none	none	none
	Phillips NE	none	none	none	none	none	none	none
	Napoleon OH	none	none	none	none	none	none	none
	Columbia PA	none	none	none	none	none	none	none
	Manheim PA	none	none	none	none	none	none	none
	Janesville WI	none	none	none	none	none	none	none
		none	none	none	none	none	none	none
93-127-01N	Waimea Kaui HI	none	none	none	none	none	none	none

Year: 1993/1994

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
93-237-054N	Waimea Kaui HI	none	none	none	none	none	none	none
93-238-01N	Santa Isabel PR	none	none	none	none	none	none	none

Year: 1994

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
94-080-04N	Henderson KY	(see *)	none	none	none	none	none	none
	Morganfield KY	none	none	none	none	none	none	none
	Hampton IA	none	none	none	none	none	none	none
	Kanawha IA	none	none	none	none	none	none	none
	Lone Tree IA	none	none	none	none	none	none	none
	Ogden IA	none	none	none	none	none	none	none
	Ottumwa IA	none	none	none	none	none	none	none
	Shennandoah IA	none	none	none	none	none	none	none
	Washington IA	none	none	none	none	none	none	none
	Webster City IA	none	none	none	none	none	none	none
	Clinton IL	none	none	none	none	none	none	none
	Hildalgo IL	(see *)	none	none	none	none	none	none
	Mt. Vernon IL	none	none	none	none	none	none	none
	Rochelle IL	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	St. Joseph IL	none	none	none	none	none	none	none
	Springfield IL	none	none	none	none	none	none	none
	Bremen IN	none	none	none	none	none	none	none
	Crawfordsville IN	none	none	none	none	none	none	none
	Greensburg IN	none	none	none	none	none	none	none
	Blissfield MI	none	none	none	none	none	none	none
	Lansing MI	none	none	none	none	none	none	none
	Maryville MO	none	none	none	none	none	none	none
	Fairbury NE	none	none	none	none	none	none	none
	Franklin NE	none	none	none	none	none	none	none
	Phillips NE	none	none	none	none	none	none	none
	Arcanum OH	none	none	none	none	none	none	none
	Napoleon OH	none	none	none	none	none	none	none
	W. Liberty OH	none	none	none	none	none	none	none
	Janesville WI	none	none	none	none	none	none	none
	Madison WI	none	none	none	none	none	none	none
94-80-05N	Fulda MN	none	none	none	none	none	none	none
	Hayfield MN	none	none	none	none	none	none	none
	Kenyon MN	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	Stanton MN	none	none	none	none	none	none	none
94-112-01N	Kalaheo, Kaui HI	none	none	none	none	none	none	none

* For these two sites in this year, slightly more Stewart's wilt susceptibility was observed. The differences were small, were not observed at the other sites or during other years, and additional evaluations are being made.

Disease and Pest Susceptibilities:

As indicated in studies presented in section **III**, and discussed above, Northrup Kings Co.'s Btk corn is significantly less susceptible to European corn borer, southwestern corn borer, corn earworm, and fall armyworm; however, no consistently significant differences are observed in susceptibility to other common corn pests and diseases, as indicated in Table V1. At two sites, of 35 total test sites, slightly more Stewart's wilt was observed in one year. The differences were small and were not observed at the other sites, or during other years. Considering the large number of characteristics monitored in a backcross conversion program such as this, it is not unexpected that one or more characteristics may show some minor differences from the original elite line during this process. Additional studies are planned for this year to look at this characteristic more closely.

As discussed in section **II. Description of the Biology of the Nonmodified Recipient Plant**, it is not expected that the regulated article will have any greater plant pest risk due to these characteristics.

Expression of the Gene Products:

Two genes are expressed within the Bt11 derived corn lines. The truncated CryIA(b) gene produces the Btk protein, which is responsible for the demonstrated resistance to Lepidopteran insect pests; and the PAT gene produces the PAT enzyme, which is used solely to select for the presence of the closely linked CryIA(b) gene, as part of the breeding program. (See **Inserted Genetic Material and Its Products** in section **IV. Description of Differences in Genotype Between Regulated Article and Nonmodified Recipient Organism.**)

Btk Protein

Information provided in section **III. Relevant Experimental Data** as Study K: Level of Btk Protein in Transgenic Plant Tissues During Plant Life Cycle, determines the levels of Btk protein present in various transgenic Bt11 corn tissues at different times in the plant life cycle. Highest Btk protein concentrations were found in the leaf tissue (to 168 ng Btk protein/mg plant protein at 25 days post planting). Generally, higher levels were detected at the younger stages of tissue development. Btk protein was detected in all plant tissues sampled. The level of Btk protein decreased as the plant reached full maturity and the tissues became senescent (to 0.4 ng Btk protein/mg plant protein in kernels at 119 days post planting).

PAT Enzyme

Studies performed by AgrEvo USA Co. and referenced herein (APHIS Petition No. 94-357-01p from AgrEvo USA Co., pp. 30-32) determine the levels of the PAT enzyme present in their corn lines transformed with the same PAT gene construction as in the Northrup King Co. Bt11 corn line. Figure N1, within Study N: Detection of Enzymatic Activity of Phosphinothricin Acetyl Transferase

(PAT) in Bt11 Corn, of section **III. Relevant Experimental Data** of this petition, demonstrates the presence of the PAT protein in the Bt11 corn line. Since the same PAT gene construction was used in the Northrup King Co. and AgrEvo USA Co. corn lines, and since similar levels of the herbicide, glufosinate, are used to select PAT gene containing plants in the field, it is expected that the levels of PAT present in Northrup King Co. line Bt11 are in the same range as that demonstrated for the AgrEvo lines tested in the above referenced studies.

New Enzymes:

The only new enzyme introduced is phosphinothricin acetyl transferase (PAT). The PAT enzyme catalyzes the acetylation of phosphinothricin which prevents the herbicide, glufonsinate, from inhibiting the plant enzyme, glutamine synthetase (see *Streptomyces viridochromogenes* in section **IV. Description of Differences in Genotype Between Regulated Article and Nonmodified Recipient Organism**). Therefore, an application of glufonsinate enables the selection of individual plants expressing the PAT gene and thus carrying the linked Btk gene. Once the PAT enzyme is established as a homozygous trait in the corn, glufonsinate will no longer need to be used as a selection tool.

Changes to Plant Metabolism:

It is expected that no significant changes to plant metabolism would occur as a result of the genetic modifications made to the Bt11 corn line. This is confirmed in studies performed at Northrup King Co. (see section **III. Relevant Experimental Data**) and the observations of our professional staff.

Weediness and Impact on Weediness of Any Other Plants With Which It May Interbreed:

These issues have been discussed in significant detail in section **II. Description of the Biology of the Nonmodified Recipient Plant**. Corn cannot maintain itself outside of cultivation as a weed, and it has no weedy relatives within the US corn belt with which it could interbreed; thus, it is not expected that the regulated article will have any greater risk for these characteristics. In addition, no weediness differences were observed during the Btk conversions and field trials, as indicated in Table V1.

Impact on Agricultural or Cultivation Practices:

It is expected that there will be no adverse impacts on agricultural or cultivation practices as the result of the use of the regulated article. In fact, Northrup King Co. expects significant benefits from the use of the Bt11 line of corn. These benefits have been presented in section **I. Introductory Discussion**.

It should be noted that the Bt11 derived corn lines, which are the subject of this petition will not be marketed as herbicide resistant.

Effects on Non-Target Organisms:

The overall toxic effect of a compound on a particular organism is the result of two factors: the inherent toxicity of the compound to the particular organism, and the exposure level of the organism to the compound. The toxicity of the Btk protein to specific groups of organisms will be discussed in more detail in the sections following this one.

As determined in Study K: Level of Btk Protein in Transgenic Plant Tissues During Plant Life Cycle, Study L: Quantity of Btk Protein Present in an Acre of Transgenic Corn (Bt11), and Study M: Fate of Btk Protein in Transgenic Plant Material and Soil, of section **III. Relevant Experimental Data**, and suggested in the scientific literature (21, 24, 31, 37), the likely level of exposure to non-target organisms, beneficial insects, soil organisms, birds, fish, aquatic invertebrates, and mammals, by transgenic plant-produced Btk protein, is very low, and is reduced additionally due to the proteinaceous nature of the Btk toxin. Although relatively stable when compared to other proteins, the Btk toxic protein fragment still demonstrates the inherent instability that is characteristic of proteins, especially when exposed to the extracellular environment (2, 11). Indeed, this inherent instability has been a major limiting factor in the usefulness of conventional Bt microbials as insecticides (15).

An additional exposure consideration is the fact that, unlike applied insecticides, the exposure level cannot be greater than that observed in the plant tissues in which it is most highly expressed. For Northrup King Co. Bt11 corn, this is in actively growing, green leaf tissue and the highest average sample value observed was 0.33 µg Btk protein/g fresh wgt. (See section **III. Relevant Experimental Data**, Study K: Level of Btk Protein in Transgenic Plant Tissues During Plant Life Cycle.)

Non-Target and Beneficial Insects

Given the origin of the gene encoding the insecticidal expression product to be covered under this registration, and the general lack of adverse effects on non-target organisms associated with the use of *Bacillus thuringiensis* biological pesticides, (6, 9, 10, 16, 18, 23, 27, 30, 35), it is expected that any exposure that may occur to non-target insects will not have a significant adverse effect.

A number of non-target insect studies are available which confirm this expectation. The studies were conducted by Monsanto Company using a trypsin-resistant fragment of the Btk HD-1 parasporal crystal protein produced in *Escherichia coli*. The studies have been submitted to the EPA in support of registration of the Btk protein and have been referenced in a Petition for Determination of

Nonregulated Status, submitted to the USDA by Monsanto Company, Docket No. 95-041-1. These studies are referenced for this petition and listed below with their EPA MRID numbers.

The studies are:

Evaluation of the Dietary Effects of Purified Btk Endotoxin Proteins on Honey Bee Larvae, MRID #43439202.

Evaluation of the Dietary Effects of Purified Btk Endotoxin Proteins on Honey Bee Adults, MRID #43439203.

Activated Btk HD-1 Protein: A Dietary Toxicity Study with Green Lacewing Larvae, MRID #43468003.

Activated Btk HD-1 Protein: A Dietary Toxicity Study with Parasitic Hymenoptera, MRID #43468004.

Activated Btk HD-1 Protein: A Dietary Toxicity Study with Ladybird Beetles, MRID #43468005.

Soil Organisms

There is an extensive body of literature which documents the highly specific nature of the toxic activity of the Btk proteins, especially that from the CryIA(b) gene, for lepidopteran insects, including those which have demonstrated the safety for non-target soil organisms (4, 6, 14, 19, 26, 29, 30, 38). Additionally, plant residue and soil degradation studies (see section **III. Relevant Experimental Data**, Study L: Quantity of Btk Protein Present in an Acre of Transgenic Corn (Bt11) and Study M: Fate of Btk Protein in Transgenic Plant Material and Soil) demonstrate a low level of exposure to the Btk protein from Bt11 plant residue for soil organisms.

All of the above suggest that the levels of Btk protein from Bt11 plant material to which soil organisms would be exposed would be minimal and no adverse effects are expected.

Birds

As argued above for Soil Organisms, a significant body of literature has demonstrated the specificity of the toxic activity of the Btk proteins for lepidopteran insects, and the lack of significant toxicity for non-insects, and specifically for vertebrates (33). This view is supported by a study submitted to EPA in support of registration of the Btk protein, A Dietary Toxicity Study with MON 80137 Meal in the Northern Bobwhite, EPA MRID #43533205, and now referenced in support of this petition.

Additionally, levels of exposure from Bt11 corn would be low. The level of Btk protein produced in homozygous, transgenic corn grain was determined to be 4.76 µg/g. This was determined in a study submitted to EPA in support of registration and titled, Determination of Levels of Plant Produced *Bacillus thuringiensis kurstaki* HD-1 Protein in Transgenic Maize, EPA MRID #43397201, and Appendix G.

All of the above argue that the Btk protein is non-toxic to birds and no adverse effects are expected.

Fish

Arguments, and associated references, for lack of toxicity to fish, based on the known toxicity range of Btk products are the same as those presented above for Birds. Additionally, arguments for lack of exposure are even more persuasive, given the fact that it would be unlikely that fish in their natural environment would be exposed to Bt11 plant material. Thus, no adverse effects are expected on fish.

Aquatic Invertebrates

Few studies have shown toxicity to aquatic invertebrates by the *kurstaki* strain of *B. thuringiensis* (30), and arguments, with associated references, for lack of toxicity to these non-target organisms are the same as those presented above. Additionally, it is expected that levels of exposure to aquatic organisms from Btk protein in Bt11 corn tissue would be low. Pollen would be the only likely plant material to provide exposure and the levels of Btk protein, at ~ 0.125 µg/g fresh weight, are extremely low. (See section **III. Relevant Experimental Data**, Study K: Level of Btk Protein in Transgenic Plant Tissue During Plant Life Cycle, and assume pollen is 10% protein.)

From the lack of observed toxicity to a large number of aquatic invertebrates, and especially from low levels of expected exposure in aquatic environments, it is expected that Bt11 corn will not have a significant adverse effect on aquatic invertebrates.

Mammals

Since there was no observed toxicity in an acute oral toxicity study in mice (submitted to EPA in support of registration of the Btk protein, also referenced in Monsanto Company's submission to USDA of 30 March 1995, and titled, Acute Oral Toxicity Study of *Btk* HD-1 Tryptic Core Protein in Albino Mice, EPA MRID #43468001), and the scientific literature (33, 34) supports this, no toxicity is expected in mammals.

Endangered Species

No endangered or threatened lepidopteran insects, as listed in 50CFR 17.11 and 17.12, feed on corn plants; and as indicated above, birds, fish, and mammals are not within the toxic specificity range of the Btk protein.

Indirect Plant Pest Effects on Other Agricultural Products:

No indirect plant pest effects are expected as the result of the use of the regulated article.

Transfer of Genetic Information to Organisms with which It Cannot Interbreed:

Natural methods of genetic exchange within the corn family are discussed in section **II. Description of the Biology of the Nonmodified Recipient Plant.** It will not be possible for the regulated article to transfer genetic information to organisms with which it cannot interbreed.

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INFORMATION UNFAVORABLE TO THE PETITION

Unfavorable information: NONE.

We are aware of no information that could be deemed unfavorable to this Petition for Nonregulated Status for Bt11 derived lines of insect protected corn expressing the CryIA(b) gene from *Bacillus thuringiensis* var. *kurstaki*.

APPENDIX A

USDA FIELD REPORTS

1992, 1993, 1994 Trials

"Btk Corn"

Northrup King Co.
7500 Olson Memorial Highway
Golden Valley, MN 55427

22 pages + cover

Northrup King Company

1993/1994 Transgenic "Btk Corn" Field Trial Results

Biotechnology Permit/Notification No's.
93-014-03, 93-127-01N, 93-237-054N,
93-238-01N, 94-069-01N, 94-080-04N,
94-80-05N, and 94-112-01N

submitted by:
Diana G. Williams

Northrup King Co.
7500 Olson Memorial Highway
Golden Valley, MN 55427

July 13, 1995

This report summarizes 1993/1994 field trials and nurseries with corn ("Btk corn") carrying a gene of the soil microorganism *Bacillus thuringiensis* var. *kurstaki*. During 1993, field evaluations on the efficacy of the Btk protein, yield and other agronomic traits were carried out within the states of KY, IA, IL, MN, NE, OH, PA and WI under USDA permit no.93-013-03. Nurseries were conducted within the states of IL and MN under the same permit and in HI under notification nos. 93-127-01N and 93-237-054N and in Puerto Rico under notification number 93-238-01N. During 1994, field evaluations on the efficacy of the Btk protein, yield and other agronomic traits were carried out within the states of KY, IA, IL, IN, MI, MO, NE, OH and WI under notification no. 94-080-04N, within MN under notification no. 94-80-05N and within MS under notification no. 94-069-01N. A nursery was carried out within HI under notification no. 94-112-01N.

Each of the trials and nurseries covered descendants of Northrup King's originally tranformed corn (transformation event Bt10 or Bt11), which was described in detail within the application for permit no. 93-013-03 as well as previously filed applications.

Information presented herewith or referenced to material within Northrup King's Petition for Determination of Nonregulated Status For Insect Protected Corn (*Zea mays* L. Expressing the CryIA(b)) Gene from *Bacillus thuringiensis* var. *kurstaki* summarizes the results of work conducted under the above permits and notifications.

Included on pages 9 - 15 is a summary of the 1993 evaluation of Btk corn resistance to European corn borer (ECB). (Also included within the information are the results of studies conducted using a different gene construct which was covered under a cooperator's permit.) Plants genetically altered to produce *Bacillus thuringiensis* var. *kurstaki* protein provided excellent control of European corn borer larvae throughout the growing season. Due to poor weather, ECB damage intensity was not as heavy in non-transformed controls as desired, but Btk corn plants were significantly less damaged (nearly immune) than non-transformed controls in all trials. Thus, it appeared that transgenic Btk corn will significantly reduce losses to the European corn borer.

In 1994, a series of efficacy trials were also conducted. In addition to evaluating efficacy against European corn borer, a few trials were established to assess efficacy against southwestern corn borer, fall armyworm and corn earworm. The results of these trials are summarized within Section III, Studies B, C, D, E and F, pages 26 - 43, of Northrup King's Petition for Determination of Nonregulated Status For Insect Protected Corn, 13 July, 1995. Btk hybrids showed excellent potential for assisting in the control of fall armyworm and southwestern corn borer. Trials will be repeated in 1995. Based upon a first year study, it appears that Btk corn hybrids display high levels of resistance to the corn earworm under artificially infested field conditions. These trials will also be repeated in 1995.

Inoculated trials conducted to assess Btk hybrid efficacy against European corn borer confirmed the results of the two previous years of field research and indicated that the Btk hybrids provide excellent season-long control of European corn borer. Btk hybrids appeared as normal and healthy with no visually apparent differences compared to non-Btk hybrids other than the lack of ECB damage. Observations were also made of Btk hybrids naturally infested with ECB. The results correlated well with observations taken in artificially infested ECB trials conducted over the past three years. The Btk hybrids provided excellent control of naturally occurring European corn borer.

In addition to evaluating the resistance of Btk corn against a number of significant pests of corn, additional characteristics were evaluated to determine whether or not significant differences existed between Btk converted and original elite corn lines. Traits evaluated in 1993 and 1994 included disease susceptibility, insect susceptibility (other than lepidoptera), weediness characteristics, stand, phenotype, potential for adverse affects on soil organisms and beneficial insects. The attached chart summarizes the results of these observations at the research and nursery sites established under the 1993 and 1994 permits and notifications. This information is also presented and discussed in greater detail within Section V, Description of the Phenotype of the Regulated Article, pages 68 - 79 of Northrup King's Petition for Determination of Nonregulated Status dated 13 July, 1995. Within that petition, Section III, Study A: Analysis of Effects of Btk Conversion on Phenotypic and Yield Attributes of *Zea mays* L. is a summary of additional evaluations made in 1994 on the lines which Northrup King intends to commercialize.

Based upon evaluations taken of field trials of Btk corn conducted in 1993 and 1994, no meaningful differences were seen between Btk corn hybrids and related non-Btk Northrup King hybrids, with the exception of resistance to European corn borer and other lepidoptera, and no plant pest characteristics were observed.

Table V1. Comparison of Btk Converted and Original Elite Corn Lines for Various Characteristics.

Comments indicate the degree to which significant differences were observed for the seven characteristics listed.

Year: 1993

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
93-014-03	Henderson KY	none	none	none	none	none	none	none
	Morganfield KY	none	none	none	none	none	none	none
	Goerge IA	none	none	none	none	none	none	none
	Hampton IA	none	none	none	none	none	none	none
	Ottumwa IA	none	none	none	none	none	none	none
	Shennandoah IA	none	none	none	none	none	none	none
	Washington IA	none	none	none	none	none	none	none
	Webster City IA	none	none	none	none	none	none	none
	Hildalgo IL	none	none	none	none	none	none	none
	Mt. Vernon IL	none	none	none	none	none	none	none
	Peoria IL	none	none	none	none	none	none	none
	Rochelle IL	none	none	none	none	none	none	none
	St. Joseph IL	none	none	none	none	none	none	none
	Springfield IL	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	Hayfield MN	none	none	none	none	none	none	none
	Kenyon MN	none	none	none	none	none	none	none
	Stanton MN	none	none	none	none	none	none	none
	Fairbury NE	none	none	none	none	none	none	none
	Phillips NE	none	none	none	none	none	none	none
	Napoleon OH	none	none	none	none	none	none	none
	Columbia PA	none	none	none	none	none	none	none
	Manheim PA	none	none	none	none	none	none	none
	Janesville WI	none	none	none	none	none	none	none
		none	none	none	none	none	none	none
93-127-01N	Waimea Kaui HI	none	none	none	none	none	none	none

Year: 1993/1994

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
93-237-054N	Waimea Kaui HI	none	none	none	none	none	none	none
93-238-01N	Santa Isabel PR	none	none	none	none	none	none	none

Year: 1994

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
94-080-04N	Henderson KY	(see *)	none	none	none	none	none	none
	Morganfield KY	none	none	none	none	none	none	none
	Hampton IA	none	none	none	none	none	none	none
	Kanawha IA	none	none	none	none	none	none	none
	Lone Tree IA	none	none	none	none	none	none	none
	Ogden IA	none	none	none	none	none	none	none
	Ottumwa IA	none	none	none	none	none	none	none
	Shennandoah IA	none	none	none	none	none	none	none
	Washington IA	none	none	none	none	none	none	none
	Webster City IA	none	none	none	none	none	none	none
	Clinton IL	none	none	none	none	none	none	none
	Hildalgo IL	(see *)	none	none	none	none	none	none
	Mt. Vernon IL	none	none	none	none	none	none	none
	Rochelle IL	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	St. Joseph IL	none	none	none	none	none	none	none
	Springfield IL	none	none	none	none	none	none	none
	Bremen IN	none	none	none	none	none	none	none
	Crawfordsville IN	none	none	none	none	none	none	none
	Greensburg IN	none	none	none	none	none	none	none
	Blissfield MI	none	none	none	none	none	none	none
	Lansing MI	none	none	none	none	none	none	none
	Maryville MO	none	none	none	none	none	none	none
	Fairbury NE	none	none	none	none	none	none	none
	Franklin NE	none	none	none	none	none	none	none
	Phillips NE	none	none	none	none	none	none	none
	Arcanum OH	none	none	none	none	none	none	none
	Napoleon OH	none	none	none	none	none	none	none
	W. Liberty OH	none	none	none	none	none	none	none
	Janesville WI	none	none	none	none	none	none	none
	Madison WI	none	none	none	none	none	none	none
94-80-05N	Fulda MN	none	none	none	none	none	none	none
	Hayfield MN	none	none	none	none	none	none	none
	Kenyon MN	none	none	none	none	none	none	none

Permit/ Notification #	Site	disease sucept.	insect sucept.	weediness char.	stand	plant phenotype	soil organisms	beneficial insects
	Stanton MN	none	none	none	none	none	none	none
94-112-01N	Kalaheo, Kaui HI	none	none	none	none	none	none	none

* For these two sites in this year, slightly more Stewart's wilt susceptibility was observed. The differences were small, were not observed at the other sites or during other years, and additional evaluations are being made.

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Northrup King Company
1993 Transgenic Bt-Corn Field Trial Results

Introduction:

To confirm results of 1992 field trials, corn genetically engineered to produce protein from *Bacillus thuringiensis* was planted at a total of 25 sites in 1993. Observation plots were planted at each of 25 yield trial sites throughout the corn belt. These plots were not artificially infested, but transgenic entries were observed for any signs of natural European corn borer, *Ostrinia nubilalis* Hübner (ECB), damage. No significant ECB feeding damage was found at any of these sites. Therefore, the remainder of this report describes only artificially infested trials.

Trials artificially infested with European corn borers were planted at three northern cornbelt sites, Webster City, IA, Stanton, MN, and Janesville, WI, and three mid-central cornbelt sites, St. Joseph, IL, Washington, IA, and Phillips, NE. The primary test objective was to determine the efficacy of transgenic corn against European corn borer larvae. Transgenic progeny plants resulting from five distinct transformation events were tested in these trials.

Methods and Materials:

Principal Investigators:

Mr. Jonathan Sagers
Entomology Department Head
Northrup King Company
317 330th Street
Stanton, MN 55018-4308
(507) 663-7629
(507) 645-7519 (FAX)

Dr. David Mies
Special Projects Coordinator
Northrup King Company
Box 710
St. Joseph, IL 61873
(217) 469-2746
(217) 469-2407 (FAX)

Experimental Design:

- Randomized complete block experimental design.
- Trials containing truncated HD-1 were planted at a total of six sites; three northern maturity trial sites at Stanton, MN, Webster City, IA, and Janesville, WI; three mid-central maturity sites at St. Joseph, IL, Washington, IA, and Phillips, NE. Two trials containing full-length HD-1 progeny were planted at St. Joseph, IL.
- Planting density was 35 non-transgenic seeds per row (35 seeds/rep) or 70 transgenic seeds per row (70 seeds/rep) in 17-foot rows. Rows were spaced 30 inches apart.
- Trials were separated into two distinct groups. One set of trials contained only hybrid F_1 seed and the other set of trials contained only inbred seed in various stages of advance. Full-length HD-1 trials contained only inbred seed in various stages of advance.
- Transgenic plants resulting from five distinct transformation events were tested. Truncated events were designated Bt10 or Bt11, and full-length events were designated "Dave", "Greg", or "Quincy" as is reflected in all subsequent documentation.
- Two non-Bt inbred corn lines were included as controls in inbred trials. Two to four commercial non-Bt hybrid controls were included in hybrid trials.

- Progeny transformed using the truncated version of HD-1 were sprayed with Basta (Ignite™) herbicide once between days 14-21 post-planting. Progeny transformed using the full-length version of HD-1 were sprayed with Glyphosate (Roundup™) 41 days post-planting or PCR screened. Herbicide resistance genes were linked with HD-1 genes as phenotypic selectable markers.

Precautions To Minimize Potential Outcrossing Events:

- Transgenic corn was surrounded on all sides by 100 feet (25 rows) of non-transgenic border corn to act as a pollen sink. Border corn was destroyed after all transgenic pollen was shed but prior to seed set by mowing down plants and/or incorporating entire plants into the soil with a disk.

Planting Dates:

- Trials were planted in accordance with standard agronomic practices for each geographic area. This helped assure normal growth and development of the corn plants. Trials were planted by May 27, 1993 at all sites.

Agronomic Practices:

Fertilization: Normal corn growing practices were followed for soil preparation and bed formation. Soil was tested prior to planting, and fertilizer was applied to bring the nutrient level up to the level recommended for corn.
Weed Control: Normal corn growing practices were followed, using labelled herbicides. Follow-up weed control was performed by hand weeding/hoeing.
Insect Control: No insecticides were necessary to control extraneous insect pests at any site.

European Corn Borer (Ostrinia nubilalis) Damage Evaluations:

Leaf Feeding Damage (First Brood) Evaluations:

Infesting: As plants reached an average height of 22-inches extended leaf height (approximately 35 days post-planting), first applications of European corn borer (Ostrinia nubilalis) neonate larvae began. Larvae were mixed into inert carrier (ground corn cob grits) and Davis Inoculators™ were used to apply two uniform shots of approximately 20 larvae per shot into the whorl of the first 10 plants of each row. This application was repeated 3 - 4 times over a two-week period for leaf feeding damage simulation. Therefore, a total of approximately 160-200 larvae were applied to each plant.

Evaluation: At all sites, final leaf feeding ratings were assigned approximately 24 days following the first infestation date. Ratings were based on the size, shape, and numbers of *Ostrinia* feeding lesions in leaves, according to non-destructive 1-9 rating scale of Guthrie et al. (1960); 1 = no visible damage, 9 = severe leaf shredding on three or more leaves.

Stalk and Ear Damage (Second Brood Damage) Evaluation:

Infesting: As plants reached a point at which 25-30% were shedding pollen, application of *Ostrinia* neonate larvae began. At least 10 plants per row were infested with neonate larvae. On each application date, one shot of approximately 15 larvae was applied to the primary ear leaf axil and the leaf axil immediately above and below the primary ear leaf. Applications were repeated four to five times over a two week period. Therefore, a total of approximately 225 larvae were applied to each plant.

Evaluation: 50-60 days after the mid-point of second brood corn borer application, at least 10 individual plant stalks were split and corn borer tunneling in each plant was measured in inches. Plants were split from the second node below the tassel to the ground using a knife.

Trial Destruction Dates and Techniques:

Trials were destroyed as soon as grain was harvested from adjacent transgenic trials. All trials were destroyed by November 1, 1993.

All trials were destroyed by turning plant debris under the soil with chisel plows or disks. Entire plants were incorporated into the soil by making two passes, the second perpendicular to the first to maximize incorporation of plant debris into the soil.

Results:

The following tables display summarized European corn borer efficacy data generated in 1993 field trials. Table 1 displays summarized data on truncated HD-1 efficacy from multiple trial sites. Table 2 displays damage ratings collected at St. Joseph, IL, from individual full-length HD-1 entries and two truncated HD-1 entries.

Table 1. Truncated HD-1 versus European Corn Borer - Summarized Results.

Entry Description	Northern Trials (MN, WI, N. IA)	Mid-Central Trials (IL, S. IA, NE)
INBRED ECB LEAF FEEDING DAMAGE SCORES[1]:		
Bt10 Inbred Lines	1.03 a	1.00 a
Bt11 Inbred Lines	1.01 a	1.00 a
Non-Transgenic Control Inbreds - Average Leaf Damage Rating (1-9, 1 = Immune)	4.25 b (Range 3.50 - 5.00)	3.38 b (Range 3.37 - 3.39)
$LSD_{(\alpha = 0.05)}$	0.70	0.69
HYBRID ECB LEAF FEEDING DAMAGE SCORES[1]:		
Bt10 Hybrids	1.05 a	1.10 a
Bt11 Hybrids	1.00 a	1.06 a
Non-Transgenic Control Hybrids -Average Leaf Damage Rating (1-9, 1 = Immune)	3.87 b (Range 3.70 - 4.00)	3.09 b (Range 2.67 - 3.91)
$LSD_{(\alpha = 0.05)}$	0.60	0.47
INBRED ECB STALK DAMAGE SCORES[2]:		
Bt10 Inbred Lines	0.00 a	0.20 a
Bt11 Inbred Lines	0.04 a	0.07 a
Non-Transgenic Control Inbred Lines - Average Leaf Damage Rating (1-9, 1 = Immune)	2.70 b (Range 1.80 - 3.60)	2.70 b (Range 1.25 - 4.14)
$LSD_{(\alpha = 0.05)}$	0.60	1.10
HYBRID ECB STALK DAMAGE SCORES[2]:		
Bt10 Hybrids	0.10 a	0.12 a
Bt11 Hybrids	0.00 a	0.03 a
Non-Transgenic Control Hybrids - Average Leaf Damage Rating (1-9, 1 = Immune)	2.37 b (Range 2.00 - 2.60)	2.49 b (Range 1.62 - 3.51)
$LSD_{(\alpha = 0.05)}$	1.50	1.10

1 - Expressed as 1-9 score, where 1 = no damage and 9 = severe damage.

2 - Expressed as average inches of ECB tunneling per plant.

Table 2. Full-Length HD-1 versus European Corn Borer (Single Location Trials - St. Joseph, IL)

Northrup Maturity Germplasm - Trial Number 40-691		
Entry Description	Leaf Feeding Damage Score[1]	Stalk Tunneling Damage Score[2]
Greg x Inbred H	1.55 a	0.00 a
Quincy x Inbred H	--Died[3]--	--Died[3]--
Dave x Inbred H	1.24 a	0.00 a
BT11 x Inbred S	1.00 a	0.00 a
BT10 x Inbred W	1.34 a	0.00 a
Inbred H	5.57 c	0.50 c
Inbred J	3.40 b	0.21 b
$LSD_{(a=0.05)}$	0.81	0.06
Pvalue	0.0019 **	<0.0000 **
Southern Maturity Germplasm		
Entry Description	Leaf Feeding Damage Score[1]	Stalk Tunneling Damage Score[2]
Dave x Inbred J	1.44 a	0.23
Greg x Inbred J	1.13 a	0.07
Inbred J	3.69 b	2.82
Inbred P (Res. Check)	4.13 b	1.07
$LSD_{(a=0.05)}$	0.72	2.43
Pvalue	0.0019 **	0.099 ns

1 - Expressed as 1-9 score, where 1 = no damage and 9 = severe damage. Same letter following mean indicates no significant difference between treatments.

2 - Expressed as average inches of ECB tunneling per plant. Same letter following man indicates no significant difference between treatments.

3 - Quincy was susceptible to Glyphosate herbicide and all plants died.

Two distinct trials were planted; one contained full-length HD-1 in germplasm adapted to the northern cornbelt and the other contained germplasm adapted to the central cornbelt. These trials were planted on May 14, 1993. Transgenic plots were sprayed with glyphosate on June 24, 1993. Plants developed using transformation event "Quincy" were inadvertently selected with Glyphosate. They were extremely susceptible and all died. Plants using transformation event

"Dave" displayed plant stunting and abnormal growth as a result of glyphosate spray. Insect damage ratings were collected on "Dave"; however, since plants were damaged by glyphosate, results should be interpreted with care. Event "Greg" plants were PCR selected and thus exhibited no symptoms of Glyphosate damage.

Summary:

1993 field trials confirmed 1992 field results and greenhouse studies. Corn plants genetically altered to produce *Bacillus thuringiensis* subspecies *kurstaki* protein provided excellent control of European corn borer larvae throughout the growing season. Due to poor weather, ECB damage intensity was not as heavy in non-transformed controls as desired, but Bt-corn plants were significantly less damaged (nearly immune) than non-transformed controls in all trials. Thus, it appears that transgenic Bt-corn will significantly reduce losses to the European corn borer.

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1993 Nort King Company BT-Corn Insect Efficacy Experiments - Summary

Trial No	Test Description (& Person Who Generated Trial)	Test Sites	Plant Date	Gene Version	Insect/Pathogen Infested With	Total Field Acreage/Site	Total Field Acreage	Transgenic Acres	Total Transgenic Acres
40-671	North Bt-Hybrids - Observation Range (D. Mies)	10 Northern Yield Trial Sites	5/11 - 5/23/93	Truncated	Not artificially infested (Observ. vs natural pests only)	0.05	0.50	0.04	0.40
70-671	South Bt-Hybrids - Observation Range (D. Mies)	15 Southern Yield Trial Sites	4/30 - 5/26/93	Truncated	Not artificially infested (Observ. vs natural pests only)	0.05	0.75	0.04	0.60
40-689	North Bt-Hybrids vs. European Corn Borer (J. Sagers)	Stanton, MN Janesville, WI Web. City, IA	5/12/93 5/14/93 5/18/93	Truncated	European Corn Borer (artificially infested)	0.017	0.05	0.01	0.03
70-689	South Bt-Hybrids vs. European Corn Borer (D. Mies)	Phillips, NE Washington, IA St. Joseph, IL	5/3/93 5/16/93 5/13/93	Truncated	European Corn Borer (artificially infested)	0.024	0.07	0.014	0.04
40-690	Bt-Inbred ECB Trial (Sagers)	Stanton, MN Janesville, WI Web. City, IA	5/12/93 5/14/93 5/18/93	Truncated	European Corn Borer	0.029	0.09	0.024	0.07
-----	Yield Loss Study (J. Graeber)	Phillips, NE Washington, IA St. Joseph, IL	5/3/93 5/16/93 5/13/93	Truncated	European Corn Borer	0.54	1.62	0.15	0.45
70-690	Bt-Inbred ECB Trial (D. Mies)	St Joseph, IL	5/13/93	Truncated	European Corn Borer	0.009	0.009	0.005	0.005
40-691	NK/Monsanto BT ECB Efficacy Trial (Sagers)	St Joseph, IL	5/27/93	Full-Length	European Corn Borer	0.017	0.017	0.012	0.012
70-691	NK/Monsanto BT ECB Efficacy Trial (Sagers)	St Joseph, IL	5/27/93	Full-Length	European Corn Borer	0.01	0.01	0.01	0.01
TOTAL---------->							3.116		1.617

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Northrup King Company
1992 Transgenic Bt-Corn Field Trial Results

Biotechnology Permit Number 92-017-03

Northrup King Co.
7500 Olson Memorial Highway
Golden Valley, MN 55427

October 22, 1993

Northrup King Company
1992 Transgenic Bt-Corn Field Trial Results

Introduction:

Trials were conducted at two mid-western sites to determine the efficacy of field corn (Zea mays) genetically engineered to produce a protein derived from Bacillus thuringiensis subspecies kurstaki. The primary test objective was to determine the efficacy of transgenic corn against European corn borer (Ostrinia nubilalis Hübner) larvae. Transgenic progeny plants resulting from three distinct transformation events were tested in these trials. European corn borer causes extensive crop damage and loss each year. Resistant crops provide growers with a method to help reduce losses to this insect pest.

Methods and Materials:

Principal Investigators:

Dr. David Mies
Special Projects Coordinator
Northrup King Company
Box 710
St. Joseph, IL 61873
(217) 469-2746
(217) 469-2407 (FAX)

Jonathan Sagers
Entomology Department Head
Northrup King Company
317 330th Street
Stanton, MN 55018-4308
(507) 663-7629
(507) 645-7519 (FAX)

Experimental Design:

- Randomized complete block experimental design.
- Two replications per location (Stanton, MN and St. Joseph, IL).
- Planted 35 seed per row (35 seed/rep) in 17-foot rows. Rows were spaced 30 inches apart.
- Transgenic plants resulting from three distinct transformation events were tested. These progeny were designated Bt10, Bt11, and Bt13.
- Three inbred corn lines with varying degrees of natural resistance to European corn borer were included as resistant controls. Five inbred corn lines with varying degrees of susceptibility were included as susceptible controls. Some non-transgenic lines were as closely related to transgenic "lines" as possible. These were included for comparison.
- Transgenic rows were sprayed with BASTA (Ignite™) herbicide once between days 14-21 post-planting. Resistance to this herbicide was linked with the Bt-resistance gene as a phenotypic selectable marker.

Precautions To Minimize Potential Outcrossing Events:

- Transgenic corn was planted 660 feet from all other corn not included as a part of this experiment.
- Test plots were surrounded with six border rows of non-transgenic hybrid "trap" corn whose nick time was very close to that of the transgenic corn to help capture pollen in these "trap" rows.
- Trials were planted approximately two weeks after surrounding growers planted non-transgenic field corn in these areas.

Planting Dates:

May 21, 1992 - St. Joseph, IL
May 22, 1992 - Stanton, MN

Trial Destruction Dates:

October 8, 1992 - St. Joseph, IL
October 30, 1992 - Stanton, MN

European Corn Borer (Ostrinia nubilalis) Damage Evaluations:

Leaf Feeding Damage (First Brood) Evaluations:

Infesting: As plants reached an average height of 22-inches extended leaf height (approximately 35 days post-planting), first applications of European corn borer (Ostrinia nubilalis Hübner) neonate larvae began. Larvae were mixed into inert carrier (ground corn cob grits) and Davis Inoculators™ were used to apply two uniform shots of approximately 45 larvae per shot into the whorl of every plant in the transgenic rows and the first 10 plants of each non-transgenic row. This application was repeated according to the following schedule:

Stanton, MN Infesting Dates: 6/26, 6/29, 7/2, 7/9; Total average number of larvae applied per plant was 382.

St. Joseph, IL Infesting Dates: Approximate dates were 6/23, 6/26, 6/30, 7/2. Number of larvae applied similar to above but exact numbers not available.

Evaluation: At Stanton early ratings were assigned for leaf feeding damage on July 2nd; 6 days following the first

infestation date. Plants were designated as "Resistant" or "Susceptible" without assigning numerical ratings. At Stanton, final leaf feeding ratings were assigned on July 20, 1992, 24 days following the first infestation date. At St. Joseph no early ratings were assigned and final ratings were assigned on July 14th, approximately 21 days following the first infestation date. Final ratings were assigned prior to tassel emergence. Every artificially infested plant within each row was observed for ECB damage and ratings were assigned on a per plant basis. Ratings were based on the size, shape, and numbers of *Ostrinia* feeding lesions in leaves, according to non-destructive 1-9 rating scale of Guthrie et al. (1960); 1 = no visible damage, 9 = severe leaf shredding on three or more leaves.

Stalk and Ear Damage (Second Brood Damage) Evaluation:

Infesting: As plants reached a point at which 25-30% were shedding pollen, application of *Ostrinia* neonate larvae began. Every plant within transgenic rows was infested. In non-transgenic control rows, only the last ten plants were infested with second brood larvae so that damage between first brood and second brood applications could be differentiated. Each application, three shots of approximately 25 larvae per shot, was applied to the primary ear leaf axil and the leaf axil immediately above and below the primary ear leaf. Applications were repeated five times according to the following schedule.

Stanton, MN Infesting Dates: 8/6, 8/10, 8/13, 8/17 8/20; Total mean number of insects applied was 377 per plant.

St. Joseph, IL Infesting Dates: Approximately 7/28, 7/31, 8/4, 8/7, and 8/11. Number of larvae applied similar to above but exact numbers not available.

Evaluation: 50-60 days after the mid-point of second brood corn borer application (September 29 and 30th at St. Joseph and October 12 at Stanton) individual plant stalks were split and corn borer tunneling was measured in inches. Plants were split from the third node above the top ear to the ground using a knife.

Agronomic Practices:

Fertilization: Normal corn growing practices were followed for soil preparation and bed formation. Soil was tested prior to planting, and fertilizer was applied to bring the nutrient level up to the level recommended for corn.
Weed Control: Normal corn growing practices were followed, using labelled herbicides. Follow-up weed control was performed by hand weeding/hoeing.

Insect Control: Plots were monitored for extraneous insect pests throughout the season, and insecticides were not necessary to control extraneous insect pests at either site.

Trial Destruction:

- Both trials were destroyed by turning plant debris under the soil with chisel plows. Entire plants were incorporated into the soil by making two passes, the second perpendicular to the first to maximize incorporation of plant debris into the soil. Trial sites were sprayed with herbicide to control corn in 1993, and they have been monitored for volunteer corn throughout 1993. Volunteers have been destroyed by hand weeding.

Results:

The following table displays summarized European corn borer efficacy data generated in the 1992 field trials conducted at St. Joseph, IL, and Stanton, MN:

Damage Description	Stanton, MN	St. Joseph, IL
LEAF FEEDING (FIRST BROOD CORN BORER) DAMAGE SCORES[1]:		
Bt10	1.0	1.1
Bt13	1.0	1.1
Bt11	1.0	1.0
Transgenic Corn - Average Leaf Damage Rating (1-9, 1=Immune)	1.0	1.1
Susceptible, Non-Transgenic Control Corn - Average Leaf Damage Rating (1-9, 1=Immune),	6.9	4.8
Resistant, Non-Transgenic Control Line - Average Leaf Damage Rating (1-9, 1=Immune)	2.8	2.7
STALK DAMAGE (SECOND BROOD CORN BORER) SCORES[2]:		
Bt10	0.1	0.1
Bt13	0.2	0.5
Bt11	0.0	0.0
Average Inches Stalk Tunneling, All Transgenic Corn	0.1	0.2
Susceptible, Non-Transgenic Control Corn - Mean Inches Stalk Tunneling	4.2	3.2
Resistant, Non-Transgenic Control Line - Mean Inches Stalk Tunneling	0.2	0.4

1 - Expressed as 1-9 score, where 1 = no damage and 9 = severe damage. Approximately 382 neonate ECB larvae applied per plant.

2 - Expressed as inches of ECB tunneling per plant. Approximately 377 neonate ECB larvae applied per plant.

Of the three transgenic progeny "lines", Bt10 and Bt11 appeared to be slightly more resistant to corn borer damage than Bt13. ELISA was performed on tissues of all transgenic plants grown at Stanton to determine relative levels of Bt protein in the leaf tissues. Generally, if Bt protein was detected at a level of 3-5 ng per mg total protein, the plant was protected against ECB damage. Thus low levels of protein controlled larvae quite effectively.

Some plants failed to express any detectable Bt protein, yet they survived BASTA herbicide spray. This happened quite commonly in Bt11 progeny. In such cases, the plants apparently contained a functional herbicide resistance gene but not a functional Bt gene.

Table 1 does not include data from plants that failed to produce Bt protein in adequate levels. These plants were susceptible to leaf feeding damage by the European corn borer and skewed the data if included.

Southern assays indicated a single copy of the Bt\PAT genes in Bt11 "lines" and at least two in Bt10 "lines". Bt13 has been discontinued. No publications were made.

Summary:

These first-year field results confirmed previous greenhouse results that demonstrated corn plants genetically altered to produce Bacillus thuringiensis subspecies kurstaki protein were considerably more resistant to European corn borer damage than non-transgenic control plants. In these 1992 field trials, in spite of artificial infesting with European corn borers, stalk damage in susceptible control plants was lighter than desired. However, test results did indicate that transgenic plants were less damaged by European corn borer (nearly immune) than even the most resistant non-transgenic control corn. All greenhouse results generated to date have supported these initial field results. Expanded field testing is underway this year to verify 1992 field results.

Transgenic plants in these trials appeared normal and indistinguishable from other non-transgenic corn. No phenotypic alterations other than those intended were noted. No altered resistance/susceptibility to pathogens or non-target insects was seen in these transgenic corn plants.

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APPENDIX B
SAMPLE BLANK FORM - 1 page

Northrup King Company

1993 Transgenic Bt-Corn Field Trial Report

Permit Number:

Site(s): Indicate on the following page, to which site(s) this report applies.

Name of person completing this report:

Observations:

- **disease susceptibility:**
- **insect susceptibility** (other than ECB):
- **weediness characteristics** (e.g., volunteers):
- **stand or germination:**
- **plant phenotype:**
- **effects on normal soil organisms:**
- **effects on beneficial insects:**
- other observations:

APPENDIX C
EXAMPLE: SITE INSPECTION BOOK - 4 pages

NORTHRUP KING

SITE INFORMATION

LOCATION NUMBER: 453

NAME: TOM KRAMER

ADDRESS: (see previous page)

PHONE: (see previous page)

SITE LOCATION: (see previous page)

LAND LEASE: 4-25

DATA COLLECTION AGREEMENT: 7-5

DATE PLANTED: Section 1 & 2 on 5-3 and section 3 thru 5 on 5-4

ROW WIDTH: 30"

PLOT LENGTH: 17'

PLANTING DEPTH: 4-4

PREVIOUS CROP: Soybeans

FERTILIZER:

DRYLAND OR IRRIGATION: Dryland

PRECIPITATION:

SITE CONDITION AT PLANTING: A good seedbed, adequate moisture, slightly lumpy

SITE CONDITION AT THINNING: 6-15 thru 6-17. Healthy plants good growth, nice plot.

SITE CONDITION AT HARVEST:

HARVEST DATE:

LOCAL CONTACTS:

LOCAL MOTEL SUGGESTION:

NORTHRUP KING

SITE INFORMATION (CONTINUED):

LOCATION NUMBER: 453

COMMENTS: 12,158 Plots
Checks sent 4-19 (research and Bt)

Site checked by Dave Bubeck On 6-30. Reported Accent damage, all exposed leaves were burned. Site will be checked again at a later date.

Site was checked and staked 6-2. Visited by Perry, Bubeck, Young on 6-2. Site staked by Proehl and Bremmer. Notes taken in Pre-c section. Herbicide sensitivity ratings taken on the Pre-c's. Plants should grow out of the damage. All damage due to Accent???

Site cultivated 6-10 by Henry Proehl.

Site thinned 6-15 thru 6-17. Plot is reported as good. Plants are healthy, leaf damage (burn) was probably due to Buctril not Accent.

Spray payment submitted for 7-5.

NORTHRUP KING

TRIAL LOCATION REPORT

LOCATION NUMBER: 453

PREVIOUS CROP: Soybeans

TILLAGE METHOD: Chisel

SOIL IDENTIFICATION:
SOIL TEXTURE: Silty Clay Loam

ORGANIC MATTER: 5.2

DRAINAGE: Well, tiled

FERTILITY LEVELS:
NITROGEN (N):
PHOSPHORUS (P): 24 PPM
POTASH (K): 168 PPM

pH: 6.8

HERBICIDE PROGRAM:

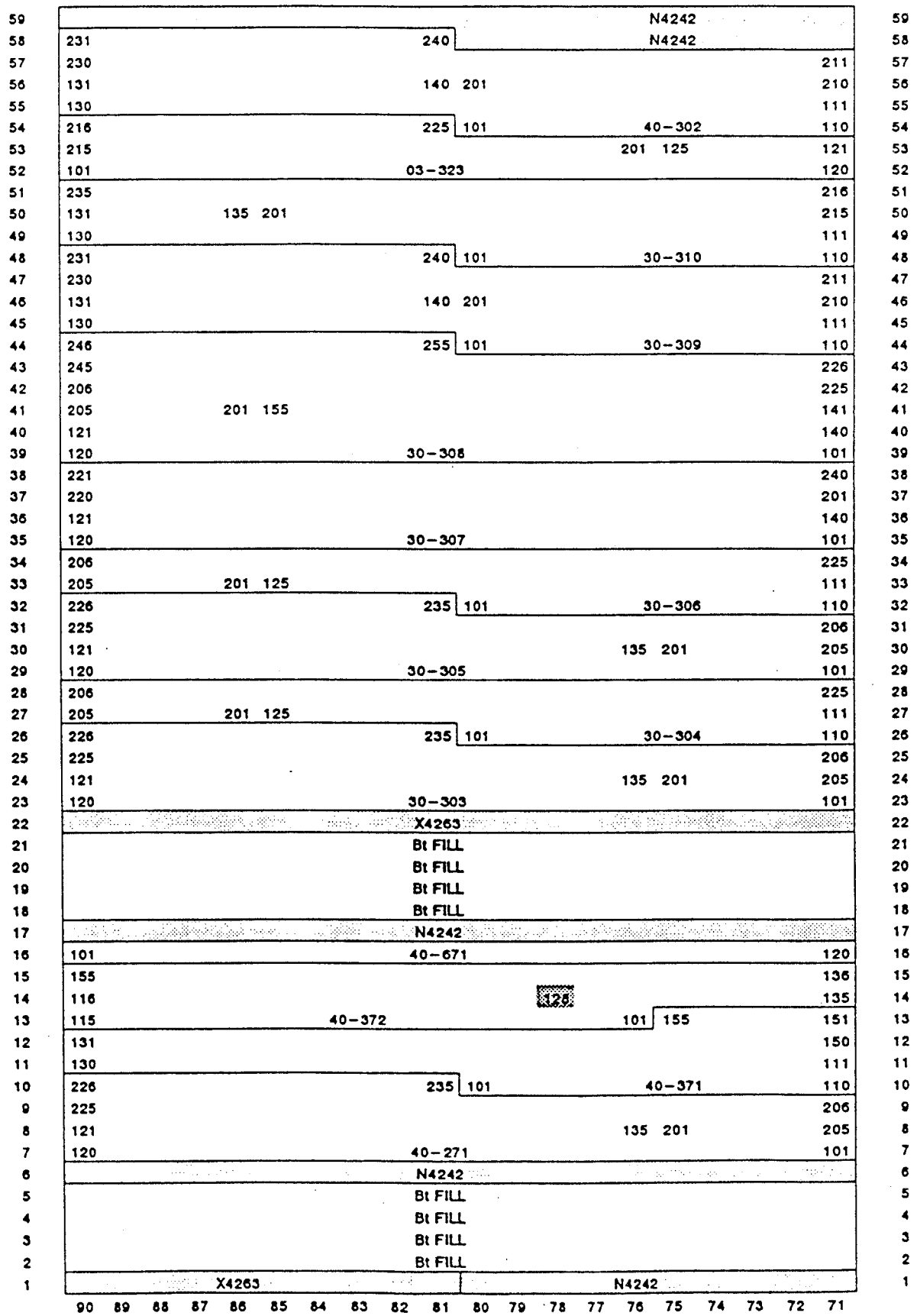
PRODUCT USED:	ACCENT	BUCTRIL/ATRAZINE	28% N
RATE APPLIED:	2/3 OZ.	2.5 PTS.	2 QTS.
WHEN APPLIED:	5-30	5-30	5-30

INSECTICIDE PROGRAM: (NONE)
PRODUCT USED:
RATE APPLIED:
WHEN APPLIED:

PREVIOUS CROP HERBICIDE PROGRAM:
PRODUCT USED: PURSUIT
RATE APPLIED: 4 OZ.
WHEN APPLIED: 6-5-93

OTHER INFORMATION THAT MIGHT BE IMPORTANT IN INTERPRETING HYBRID PERFORMANCE:

1994
HAYFIELD 453 SECTION 4



SEED LABEL - 1 page

"Notice To Grower"

This hybrid also produces a protein which increases its tolerance to the herbicide glufosinate ammonium. *Glufosinate ammonium is not registered or recommended for use on this hybrid.* If you plant a glufosinate resistant crop in the next growing season, please note that volunteer plants from this corn hybrid may not be controlled by a glufosinate ammonium herbicide.

PETITION FOR DETERMINATION OF NONREGULATED STATUS

Insect Protected Corn (*Zea mays* L.) Expressing the CryIA(b) Gene from *Bacillus thuringiensis* var. *kurstaki*

APPENDIX E

INSECT RESISTANCE MANAGEMENT

Management of Insect Pests with Insect Protected Corn: Recommended Approaches

10 pages

MANAGEMENT OF INSECT PESTS WITH INSECT PROTECTED CORN:

RECOMMENDED APPROACHES

Northup King Co.
Golden Valley, MN

Abstract

Insect protected corn, which exhibits a high level of protection to damage and yield loss by lepidopteran pests, has been developed through the expression of a *Bacillus thuringiensis* var. *kurstaki* gene [CryIA(b)] in corn. Northrup King Co. has developed recommended approaches to utilize these plants to maximize the utility and durability of these new insect control products. These approaches are being tested and will require optimization in the field during commercial introduction.

Introduction

Genetic insect resistant crops represent an important new management tool to control crop damage and loss due to insect pests. These plants offer significant benefits to the grower, the consumer and the environment. Insect resistance has been developed through the expression of a gene that produces an insecticidal protein from *Bacillus thuringiensis* (Bt) in the cells of the corn plant. The particular gene being developed by Northrup King for corn is derived from the *B.t. kurstaki* (Btk) strain. This protein is the basis of several commercially available microbial insecticides, which have been demonstrated as highly selective for lepidopteran insects, with no activity against other types of organisms such as mammals, fish, birds or non-insect invertebrates (earthworms, spiders, etc.) (EPA, 1991; EPA, 1988). In addition, this protein shows a remarkable insect specificity (Macintosh, *et al,* 1990). The Btk genes developed for corn produces a protein that is active only against certain lepidopteran larvae with no activity against other orders of insects. Importantly, this activity spectrum overlaps with several important pests of corn which include the European corn borer, corn earworm, and fall army worm.

The use of insect protected plants will provide important benefits to growers, society, and the environment (McGaughey and Whalon, 1992; Gasser and Fraley, 1989; Gould, 1988). First and foremost, these plants offer an alternative to chemical insecticides currently used to control susceptible insect pests with efficacy equal to or better than that of current control methods. The use of insect protected corn will significantly reduce the application of chemical insecticides directed at these pests. The reduction of insecticide use will have direct benefits to the grower. such as less time and effort spent on insect control and reduced exposure to chemical

insecticides.

Genetic insect resistant corn is likely to produce secondary benefits in pest control as an indirect result of the reduction in use of chemical insecticides. Chemical insecticides are relatively non-specific and have the effect of killing beneficial predatory and parasitic insects (Roush and Tingey, 1993; Van den Bosch and Stern, 1962). Because the Bt proteins produced by transformed plants are not active against these beneficial insects, populations have been shown to rise significantly in fields planted with insect protected cotton and Colorado potato beetle protected potatoes compared to nontransgenic cotton and potatoes treated with chemical insecticides (Read, *et al.*, 1992). Preserving the beneficial insect population should enhance the biological control of both target pests and non-target pests in transgenic corn fields.

The use of insect protected plants will provide important benefits to growers, society and the environment. To achieve these benefits, it is important that resistance management strategies be implemented. In this respect, these plants are no different than any other pesticide. There are two aspects of this management. First, is the development of pest management techniques that allow the farmer to maximize the ability of these plants to control target pests. In essence, this is the development of a total insect management package that will be centered around a new tool, transgenic corn expressing the Btk insecticidal protein. Second, is the development of appropriate strategies to maximize the product durability and the utility of genetic insect protection. Part of this management program is the development and implementation of strategies targeted to prevent the development of insect resistance to the Btk proteins produced in corn. Because both management aspects can affect the way in which genetically protected plants are used by the grower, these two types of management, total pest management and insect resistance management, are interconnected.

Resistance management is not an issue particular to insect protected plants, given the development of insect resistance to chemical insecticides. Scientists have addressed insect resistance for several years in laboratory and field studies. Several suggestions have been examined for resistance management in transformed plants (Everich, 1994; Roush, 1994; Sachs, 1993; Stone and Sims, 1993). As the following discussion will demonstrate, promising strategies for resistance management for insect protected plants are available and can be recommended. These strategies may be specific for each target pest and need to account for crop production and agronomic practices. It is evident, however, that genetically protected plants offer unique options for pest and resistance management that are not available with traditional pesticides.

Integrated Pest and Resistance Management with Insect Protected Corn

In many areas lepidopteran pests are the primary damaging insects of corn, so the use of Btk corn to control these pests will be a major portion of total insect control. By substituting genetically modified corn for chemical pesticides, a positive impact on overall insect management will result. Many of the details of pest management with insect protected plants can only be

determined by multi-year large scale field tests designed to incorporate these genetically modified crops into current production practices. Such field trials are in progress and are providing the data needed for developing a pest and resistance management program for these crops. These trials involve collaborations between Northrup King, other company partners, and academic and extension entomologists. They are examining the impact of insect protected plants on populations of beneficial and pest insects endemic to the crops and the impact on the use of conventional insecticides for controlling non-target pests (Reed, *et al,* 1992), the establishment of the baseline susceptibility of our insect targets to Btk protein (Stone and Sims, 1993; Everich, 1994) and the impact of mixtures of protected and non-protected plants on yield loss (Roush, 1994).

Insect protected corn will be an important addition to the available methods of controlling insect pests. The implementation of these plants is fully consistent with the goals of integrated pest management because:

a) the Btk protein produced by the corn plant is insect specific, affecting only a few targeted pest species.

b) the Btk protein is active only against insects doing damage by feeding on the plant.

c) use of the plants will reduce the application of chemical insecticides.

d) use of the plants will preserve beneficial insects, which will enhance the biological control of non-target pests.

Because pest and resistance management are interconnected, it is important to develop both of these approaches.

Combination of Insect Protected Plants with Chemical Insecticides

One aspect of the use of insect protected plants for integrated pest management in corn is the continued use of chemical insecticides. Some insecticides will continue to be used in corn for non-lepidopteran pests. If possible, these insecticides need to be chosen so as to not negatively impact beneficial arthropods, which are integral in the biological control of non-susceptible species. The combination of insect protected crops with chemical insecticides, while part of a total insect control package, is not a resistance management option for insect protected plants per se. Chemical insecticides can reduce the population size of insects selected for resistance to Btk but cannot alter the gene frequencies within this population (Roush, 1989). Alternatively, the use of genetically protected plants should positively impact current chemical insecticides by helping slow resistance development and prolonging the life of these important agricultural chemicals.

Resistance Management for Insect Protected Plants

As described above, part of managing the implementation of genetically protected plants is the design and implementation of appropriate strategies to delay or prevent the development of insect resistance to the Btk protein in corn. Described below are general approaches that will help manage resistance development in corn insect pests. It is important to note that: 1) as insect resistance development is a biological phenomenon, the rate of development is difficult if not impossible to predict and consequently, the efficacy of a strategy to delay or prevent its development may be impossible to demonstrate; 2) because of the available technology, biology of the pest, and the production practices of the crop, implementation of these strategies will be dependent on the crop and the target pest; and 3) field research must be conducted to determine the practical implementation of these strategies within current crop production practices. These strategies have been recommended by several researchers (Gould, 1988; Stone et al., 1991; McGaughey and Whalon, 1992) and are summarized briefly below and then expanded in greater detail in the next section.

Summary of Considered Resistance Management Strategies for Insect Protected Corn

- High dose expression of Btk protein in corn to control insects heterozygous for resistance alleles.
- Refugia as hosts for sensitive insects provided through non-insect protected plants or other non-modified hosts.
- Monitoring of insect populations for susceptibility to Btk protein.
- Agronomic practices that minimize insect exposure to Btk corn.
- Integrated pest management (as described above).
- Combination of multiple genes within the same corn plant, both of which are active against targeted insects but with different sites/modes of action.
- Incorporation of host plant resistance traits into insect protected corn as they are proven effective.
- Incorporation of novel proteins that provide effective control of targeted pests.

High dose expression

High dose expression for resistance management is based on three assumptions:

1) Resistance will most likely be controlled by one major locus with recessive resistance alleles (McGaughey and Beeman, 1988; Macintosh *et al,* 1991; Sims and Stone, 1991).

2) Insects developing resistance to the Btk protein will be rare initially and will almost always mate with susceptible insects giving rise to heterozygous progeny (Gould, 1986).

3) More than 95% of the heterozygous progeny will be disabled or killed by Btk corn plants with the same dose as the homozygous susceptible larvae.

The high dose expression strategy uses plant expression of Btk protein in quantities sufficient to kill those insects heterozygous for resistance to Btk (McGaughey and Whalon, 1992; Roush, 1989). This resistance strategy fits nicely with the fact that high dose expression is essential for commercial efficacy of insect protected corn. High dose expression is also necessary to maintain consistent control across environments and genotypes. We plan to evaluate and develop the high dose expression strategy.

Refugia for Sensitive Insects

Refugia means providing a refuge for sensitive insects within a population so they will not be exposed to Btk protein and not be selected for resistance. As a resistance management technique, refugia is based on the concept that control failure due to resistance is a population genetics phenomenon. Control failures are observed when the frequency of protected insects in the population reaches a critical level. Refugia supply susceptible non-selected individuals to the general population. With adequate refugia, the frequency of resistance genes will be very low and spread only very slowly through the population. Refugia is an important component of our resistance management strategies.

Refugia can be provided either within the crop or outside it. The refuge can also be planted specifically as such or exist naturally. In all of these approaches, the effectiveness of the refuge is based on those insects that survive on the refuge crop rather than its total acreage. This is an important point because, if the refuge is chemically treated, the refuge population is reduced and the amount of acreage required is increased. Examples of refugia that can be utilized are:

1) Refuge outside of the crop: Non-insect protected corn.

This type of refuge will exist in all the acres not covered by the genetically protected plants. This area will be substantial in the early years after introduction and could supply a sufficient refuge for several years. As insect protected seed becomes more available and widely grown, this refuge will be reduced. Consequently, over time, reliance on non-insect protected corn fields for refugia may be less effective.

2) Refuge outside of the crop: Non-modified crop hosts.

The European corn borer, corn earworm, and the fall army worm have many non-corn hosts which may provide an adequate refuge. In some locations corn may be the only host for at least one insect generation per season. The use of Bt microbials or other transgenic (Bt) crops will also impact their utility as a refuge for insect protected plants. This option must be evaluated carefully based on the crop, pest biology, and growing regions.

3) Refuge within the crop: Non-insect protected plants.

An "in crop" refuge of non-insect protected plants has been suggested as a simple system for insuring a refuge. For this in crop refuge, the choices are: a) random mixture of seed of insect protected and non-protected plants or b) non-insect protected plants planted within the same field.

Mixed seed lines (Btk and non-insect protected seed within the same bag) have a certain appeal due to the 'automatic' implementation. A possible problem with mixed seed arises from larvae that survive on a non-insect protected plant and migrate to a modified plant where they may be less sensitive to Btk protein because of their size. This could compromise insect control and increase selection pressure for resistance. The likelihood of this occurring is being investigated experimentally before this strategy is implemented.

Agronomic Practices

Certain agronomic practices may be recommended for insect protected plants. Effective reductions of overwintering populations of European corn borer in corn have been obtained through stalk chopping followed by plowing under crop residue (USDA, 1962). The recommendation of these strategies will be determined on a regional basis, if necessary.

Monitoring Insect Resistance

Insect resistance monitoring is an important component of any insect resistance management strategy. A baseline frequency is in development. Resistance of major target pests to Bt protein has not been detected in the field (Everich, 1994; Stone and Sims, 1993). Baseline information should be collected on all Bt products (engineered plants and Bt microbials) to know when the frequency of resistant genotypes have increased within the population. This information must be developed on regional bases over several years so that susceptibility changes in populations can be identified and validated.

Pyramiding Traits

A set of strategies for the medium and long term focus on combining multiple insecticidal

agents. The rationale is essentially the same for all of these: Expose the insects to two or more active agents with distinct modes of action at the same time, and the probability of any one insect being selected for resistance to both agents simultaneously is extremely low.

1) Combination with a Second Insect Resistance Gene

A second gene within the same plant possessing a different mode of action will significantly reduce the frequency of resistant individuals (Peferoen, 1992; Stone et al., 1991; Van Rie, 1991). Population models indicate that other alternative uses of a second gene such as seed mixture or using single genes in rotation, may be as effective as two genes within the same plant (Gould, 1988: Gould 1986). Assuming initial gene frequencies for Btk protein resistance are low, initial introduction of a product with a single Btk gene should not negatively compromise a second gene because the single gene product will be planted on limited acres in the first few years. In the medium term the best choice of second gene is an unrelated Bt gene. In the long term, the use of novel, non-Bt insecticidal genes holds great promise. This area is under active research.

2) Combination with Host Plant Resistance Traits

This is a long term strategy that fits with our classical breeding programs for insect resistance. Host plant resistance traits (HPR) used in combination with transgenic insect control needs to be insecticidally effective and not negatively impact quality or yield.

Summary

Genetically protected corn will offer great benefits in overall insect control. These plants will be developed to fit within existing pest management practices. With proper management and implementation, the development of insect resistance to Btk will not be a technical or commercial problem that will limit the value or efficacy of these products. Northrup King Co. in cooperation with industry and academic collaborators has developed a package of strategies that will help effectively manage the potential development of insect resistance to Btk corn. The details of this program and its incorporation into existing pest management programs will be further developed and optimized in the field in the coming years.

Many aspects of the use of genetic insect protection in plants and the implementation of resistance management strategies are unique to these products as compared to traditional chemical or microbial insecticides. For example, the use of refugia and the incorporation of multiple resistance traits through molecular biology or by plant breeding are aspects that are ideally suited to transgenic protection of plants. This ability to utilize new methods for pest and resistance management make genetically modified plants a critical component for successfully managing insect pests in the future.

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APPENDIX F

14 pages

VOLUME 6

TOXICITY/ALLERGENICITY CONSIDERATIONS PHOSPHINOTHRICIN ACETYL TRANSFERASE

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June 7, 1994

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this volume on the basis of its falling within the scope of FIFRA 10(d)(1)(A), (B), or (C).

Company: Northrup King Co.

Company Agent: Diana G. Williams

Diana G. Williams
Manager, Government Regulations

Date: August 22, 1994

As previously described within MRID NO. 43130801, Product Characterization, the PAT marker gene was isolated from a phosphinothricin-resistant strain of *Streptomyces viridochromogenes*(1-4), a common soil bacterium first identified in 1914 (6). The PAT gene codes for an acetyl-transferase enzyme (phosphinothricin acetyl-transferase, or PAT), which inactivates the phosphinothricin molecule by acetylation.

Also as previously described, the PAT gene is linked with the Btk HD-1 gene and is expressed throughout the Btk corn plant. Exposure to the PAT enzyme will be possible through consumption of the Btk corn. Therefore, to address whether the PAT enzyme is potentially a toxic agent or allergen, the following investigations were made.

<u>Acetyl-Transferases as a Class</u>

Acetyl transferases are a class of enzymes common to all bacterial, plant and animal cells. Acetyl transferases play a central role in both the synthesis and oxidation of fats (10).

A wide range of acetyl transferases has been documented with differing substrate specificities. Acetyl transferases represent one of the six major families of enzymes which metabolize drugs in humans (11). Enzymatic acetylation has been reported as a defense against carcinogens (12), and the ability to acetylate chloramphenicol is responsible for the resistance of some *Salmonella* strains to chloramphenicol (13).

Since virtually all cells contain acetyl transferases, these enzymes are a natural component of the human diet.

To determine whether phosphinothricin acetyl transferase is likely to pose any adverse toxicological effects, a literature search for reports of toxicity or allergenicity associated with acetyl-transferases was conducted. The search screened for reports in which "Acetyl Transferase" occurred with one or more of the following terms:

"Allergen" "Toxin" "Venom" "Human" "Plants"

The following biological, chemical and medical databases were searched: AGRICOLA, BIO Business, Biological Abstracts, CAB International, Chemical Engineering Abstracts, Life Sciences Collection, MEDLINE and NIOSHTIC. No reports were found of toxicity or allergenicity associated with acetyl transferase.

The Phosphinothricin Acetyl Transferase Enzyme

On the basis of the above cited search, acetyl transferases as a group appear devoid of reported mammalian toxicity or allergenicity. To address whether the specific acetyl transferase expressed within the corn covered under this application mimics a known toxin or allergen in its amino acid composition, the PC/GENE protein database was scanned for the existence of proteins with sequences related to that of the PAT enzyme (Attachment 1, pages 8 - 14).

Only one enzyme showed significant (@ 95%) homology, the BAR protein from *Streptomyces hygroscopicus,* which is also a phosphinothricin producing organism. BAR, like PAT, is itself a phosphonothricin acetyl-transferase (5).

No other protein among the 26,706 entries in the database has significant homology to the PAT gene. The next closest match (an oxygenase from *Pseudomonas*) has less than 12% homology. The PAT gene, therefore, shares no significant sequence homology with any protein toxins, bacterial enterotoxins, allergens or venoms in the database.

The Donor Organism: *Streptomyces viridochromogenes*

The genus *Streptomyces* is known to contain a very small number of species capable of causing disease in humans and animals, primarily infections of wounds (7). These "mycetoses" are common only in Africa and Latin America, and of these, only about 5% involve *Streptomycete* species. Gordon states "...most Streptomyces species are not known to incite human or animal disease and are not ordinarily found in clinical specimens" (8). This is to be expected, since most soil *Streptomycetes*, like *viridochromogenes*, are aerobic saprophytes which break down dead organic matter in the soil.

The particular *Streptomycete* from which the PAT gene was isolated, *Streptomyces viridochromogenes*, is not a pathogen of man, animals or plants (7-9). A search of the recent literature in eight databases failed to produce any reports of involvement in human or animal disease for this organism. The databases searched were: AGRICOLA, BIO Business, Biological Abstracts, CAB

International, Chemical Engineering Abstracts, Conference Papers Index, Life Sciences Collection and MEDLINE.

Since Streptomyces viridochromogenes has no known pathogenic potential, there is no reason to expect that transferring one of its genes will move undesirable, pathogenic or toxic properties to the recipient organism.

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Attachment 1

PC/GENE DATA BASE SCAN

===11-MAR-1994===PC/GENE===

****
FAST SCAN FOR SIMILARITY TO A PROTEIN SEQUENCE *
****

Done on sequence: PATPRO.

DE PAT GENE PROTEIN
OS CORN

The total number of amino acids in this sequence is: 183.
The scan was done using a 'k-tuple' value of 1.
The 'distance' parameter is set to 4 amino acids.
The final scores were computed using the Structure-genetic matrix.
The scan was performed with all the protein sequences in data base: CDPROT23.
The number of sequences successfully scanned was 26706.
The average score in this scan with the current parameters is: 56.93855
The standard deviation is: 13.54361
The reference score of the sequence against itself is: 1098.

Table of the best scores.
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Nb	Absolute score	Relative score (%)	Sequence name	Position	Position in PATPRO
1	1038	94.5	BAR_STRHY	1 - 183	1 - 183
2	130	11.8	LT23_CAEEL	877 - 906	100 - 129
3	129	11.7	PEN3_ADE02	319 - 349	9 - 39
4	129	11.7	PEN3_ADE05	319 - 349	9 - 39
5	122	11.1	CH60_SYNP7	355 - 382	45 - 72
6	120	10.9	RIBT_BACSU	30 - 57	44 - 71
7	119	10.8	BPHC_PSEPS	43 - 70	55 - 82
8	118	10.7	PHFL_DESVH	122 - 151	64 - 93
9	117	10.6	FABD_ECOLI	184 - 212	120 - 148
10	117	10.6	YHRD_STRCO	73 - 96	70 - 93
11	113	10.2	CH60_CLOPE	359 - 382	49 - 72
12	113	10.2	PULO_KLEPN	150 - 177	43 - 70
13	112	10.2	EPD2_CARAU	149 - 175	137 - 163
14	111	10.1	P3_MOUSE	101 - 128	66 - 93
15	111	10.1	RP54_BACSU	202 - 228	42 - 68
16	110	10.0	EPD_BRARE	150 - 176	137 - 163
17	110	10.0	GAG_IPMA	659 - 683	112 - 136
18	110	10.0	TRK6_ECOLI	39 - 65	13 - 39
19	109	9.92	IE14_VZVD	296 - 319	131 - 154
20	109	9.92	RECA_BACFR	144 - 171	127 - 154

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Scoring sequence segments.
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PATPRO          1- MSPERRPVEIRPATAADMAAVCDIVNHYIETSTVNFRTEPQTPQEWIDDL
                   ::::::: :: :: ::: ::: :::::::::::::::::: :::: :::
BAR_STRHY       1- MSPERRPADIRRATEADMPAVCTIVNHYIETSTVNFRTEPQEPQEWTDDL

                   ERLQDRYPWLVAEVEGVVAGIAYAGPWKARNAYDWTVESTVYVSHRHQRL
                    ::  ::::::::: : :::::::::::::::::: ::::::: ::::
                   VRLRERYPWLVAEVDGEVAGIAYAGPWKARNAYDWTAESTVYVSPRHQRT

                   GLGSTLYTHLLKSMEAQGFKSVVAVIGLPNDPSVRLHEALGYTARGTLRA
                   :::::::::::::: :::::::::::::::::::: :::::: :: :::
                   GLGSTLYTHLLKSLEAQGFKSVVAVIGLPNDPSVRMHEALGYAPRGMLRA

                   AGYKHGGWHDVGFWQRDFELPAPPRPVRPVTQI
                   :: ::: :::::::: :: :: ::::: ::: :
                   AGFKHGNWHDVGFWQLDFSLPVPPRPVLPVTEI


PATPRO        100- LGLGSTLYTHLLKSMEAQGFKSVVAVIGLP
                   :   : : : : :   : : : : :  :
LT23_CAEEL    877- LIPSSELQTKLDKKLGAGAFGTVFAGIYYP


PATPRO          9- EIRPATAADMAAVCDIVNHYIETSTVNFRTE
                   :   : :: :  : :   : :   :   : :
  N3_ADE02    319- ENSNAAAAAMQPVEDMNDHAIRGDTFATRAE


PATPRO          9- EIRPATAADMAAVCDIVNHYIETSTVNFRTE
                   :   : :: :  : :   : :   :   : :
PEN3_ADE05    319- ENSNAAAAAMQPVEDMNDHAIRGDTFATRAE


PATPRO         45- EWIDDLERLQDRYPWLVAEVEGVVAGIA
                   :   : : :: :   :   :  : : :
CH60_SYNP7    355- ESSYDKEKLQERLAKLSGGVAVVKVGAA


PATPRO         44- QEWIDDLERLQDRYPWLVAEVEGVVAGI
                   :  : : :   ::   :  : : :  :
RIBT_BACSU     30- QQTIKDYETDTDRQLFLWKEDEDIVGAI


PATPRO         55- DRYPWLVAEVEGVVAGIAYAGPWKARNA
                   :   :  :   : :   : ::   :  :
BPHC_PSEPS     43- DSRAWRIAVQQGEVDDLAFAGYEVADAA


PATPRO         64- VEGVVAGIAYAGPWKARNAYDWTVESTVYV
                   :  :  :   :   :   :  :  : : :
  FL_DESVH    122- VGSVTTGKMLAALQKLGFAHCWDTEFTADV
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10 - nk5pttx

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PATPRO        120- KSVVAVIGLPNDPSVRLHEALGYTARGTL
                   :   :   ::   ::  : ::   :   :
. 5D_ECOLI    184- KAAGAKRALPLPVSVPSHCALMKPAADKL

PATPRO         70- GIAYAGPWKARNAYDWTVESTVYV
                   : :   :  :  ::   :: ::::
YHRD_STRCO     73- GYATSSPYRAKPAYATSVETTVYV

PATPRO         49- DLERLQDRYPWLVAEVEGVVAGIA
                   : : :: :   :   :  :  : :
CH60_CLOPE    359- DKEKLQERLAKLAGGVAVVKVGAA

PATPRO         43- PQEWIDDLERLQDRYPWLVAEVEGVVAG
                   :  :   :  :   :  :   : :  ::
PULO_KLEPN    150- PLMWAGLLFNLSATYVPLAEAVVGAMAG

PATPRO        137- HEALGYTARGTLRAAGYKHGGWHDVGF
                   :  :  :  : :   :   :   :  :
EPD2_CARAU    149- HYSLSITSCGCLPVSGSYYGDKKDLLF

PATPRO         66- GVVAGIAYAGPWKARNAYDWTVESTVYV
                   :   :   :   :   :   ::  : :
   MOUSE      101- GLLVGYSLAICLKLPVAQRRTVSIEVGV

PATPRO         42- TPQEWIDDLERLQDRYPWLVAEVEGVV
                   : :   ::   :  :   : :  :  :
RP54_BACSU    202- TIQDISDDIAALHPRPGLLFARPEQDV

PATPRO        137- HEALGYTARGTLRAAGYKHGGWHDVGF
                   :  :  :  : :   :   :   :  :
EPD_BRARE     150- HYSLSTTSCGCLTVSGSYYGEKKDLFF

PATPRO        112- KSMEAQGFKSVVAVIGLPNDPSVRL
                   : :   :  :   :  :   :  ::
GAG_IPMA      659- KKMGSSGNDSAYLVVSLNDRPKLRL

PATPRO         13- ATAADMAAVCDIVNHYIETSTVNFRTE
                   :  :   :   :  :  ::  : :  :
TRK6_ECOLI     39- AALASGYALVTIWEHMRETGKVKFSYE

PATPRO        131- DPSVRLHEALGYTARGTLRAAGYK
                   :   : : : : : :   :  : :
   '_VZVD     296- DTRPRKHDARGITPRVPGRSSGGK
```

11 - nk5pttx

```
PATPRO        127- GLPNDPSVRLHEALGYTARGTLRAAGYK
                   :  :  : :   :   :   :  :  :
R. A_BACFR    144- GDMGDNKVGLQARLMSQALRKLTSAVSK
```

Dictionary of the scoring sequences.
==========

```
BAR_STRHY
DE   PHOSPHINOTHRICIN ACETYLTRANSFERASE (EC 2.3.1.-).
OS   STREPTOMYCES HYGROSCOPICUS.

BPHC_PSEPS
DE   BIPHENYL-2,3-DIOL 1,2-DIOXYGENASE (EC 1.13.11.39) (23OHBP OXYGENASE)
OS   PSEUDOMONAS PSEUDOALCALIGENES.

CH60_CLOPE
DE   60 KD CHAPERONIN (PROTEIN CPN60).
OS   CLOSTRIDIUM PERFRINGENS.

CH60_SYNP7
DE   60 KD CHAPERONIN (GROEL HOMOLOGUE) (PROTEIN CPN60).
OS   SYNECHOCOCCUS SP. (STRAIN PCC 7942) (ANACYSTIS NIDULANS R2).

EPD2_CARAU
I    EPENDYMIN II PRECURSOR (EPD-II).
OS   CARASSIUS AURATUS (GOLDFISH).

EPD_BRARE
DE   EPENDYMIN PRECURSOR.
OS   BRACHYDANIO RERIO (ZEBRAFISH) (ZEBRA DANIO).

FABD_ECOLI
DE   MALONYL COA-ACYL CARRIER PROTEIN TRANSACYLASE (EC 2.3.1.39).
OS   ESCHERICHIA COLI.

GAG_IPMA
DE   PUTATIVE GAG POLYPROTEIN.
OS   MOUSE INTRACISTERNAL A-PARTICLE (IAP-MIA14).

IE14_VZVD
DE   IMMEDIATE-EARLY PROTEIN IE140.
OS   VARICELLA-ZOSTER VIRUS (STRAIN DUMAS) (VZV).

LT23_CAEEL
DE   LET-23 RECEPTOR PROTEIN-TYROSINE KINASE PRECURSOR (EC 2.7.1.112).
OS   CAENORHABDITIS ELEGANS.

P3_MOUSE
DE   P3 PROTEIN (FRAGMENT).
     MUS MUSCULUS (MOUSE).
```

PEN3_ADE02
DE   PENTON PROTEIN (VIRION COMPONENT III).
     HUMAN ADENOVIRUS TYPE 2.

PEN3_ADE05
DE   PENTON PROTEIN (VIRION COMPONENT III).
OS   HUMAN ADENOVIRUS TYPE 5.

PHFL_DESVH
DE   PERIPLASMIC [FE] HYDROGENASE LARGE SUBUNIT (EC 1.18.99.1) (FE
OS   DESULFOVIBRIO VULGARIS (STRAIN HILDENBOROUGH).

PULO_KLEPN
DE   PULLULANASE SECRETION PROTEIN PULO (EC 3.4.99.-).
OS   KLEBSIELLA PNEUMONIAE.

RECA_BACFR
DE   RECA PROTEIN (EC 3.4.99.37).
OS   BACTEROIDES FRAGILIS.

RIBT_BACSU
DE   RIBT PROTEIN.
OS   BACILLUS SUBTILIS.

RP54_BACSU
DE   RNA POLYMERASE SIGMA-54 FACTOR.
OS   BACILLUS SUBTILIS.

   5_ECOLI
DE   TRAK PROTEIN.
OS   ESCHERICHIA COLI.

YHRD_STRCO
DE   HYPOTHETICAL PROTEIN IN HRDD GENE REGION (ORF X) (FRAGMENT).
OS   STREPTOMYCES COELICOLOR.

===11-MAR-1994==========PC/GENE===

****
FAST SCAN FOR SIMILARITY TO A PROTEIN SEQUENCE *
****

Done on sequence: PATPRO.

DE PAT GENE PROTEIN
OS CORN

The total number of amino acids in this sequence is: 183.
The scan was done using a 'k-tuple' value of 1.
The 'distance' parameter is set to 4 amino acids.
The final scores were computed using the Structure-genetic matrix.
The scan was performed with the protein sequences listed in library: ACYL_TRN.
The number of sequences successfully scanned was 159.
The average score in this scan with the current parameters is: 67.25157
The standard deviation is: 78.18147
The reference score of the sequence against itself is: 1098.

Table of the best scores.
==========

Nb	Absolute score	Relative score (%)	Sequence name	Position	Position in PATPRO
1	1038	94.5	BAR_STRHY	1 - 183	1 - 183
2	97	8.83	AAC6_KLEPN	60 - 82	50 - 72
3	97	8.83	AAC6_SERMA	60 - 82	50 - 72
4	91	8.28	ODP2_NEUCR	328 - 346	106 - 124
5	89	8.1	ODP2_HUMAN	374 - 394	12 - 32
6	82	7.46	ARGA_ECOLI	100 - 119	124 - 143
7	82	7.46	NODL_RHILV	46 - 66	56 - 76
8	79	7.19	CHSD_PETHY	374 - 391	101 - 118
9	78	7.1	HEM1_RHIME	36 - 54	76 - 94
10	77	7.01	ODP2_AZOVI	68 - 85	62 - 79
11	77	7.01	THI1_RAT	309 - 327	64 - 82
12	77	7.01	THI2_RAT	319 - 337	64 - 82
13	75	6.83	HEM1_ECOLI	214 - 230	52 - 68
14	75	6.83	HEM1_SALTY	214 - 230	52 - 68
15	74	6.73	ODP2_BACSU	94 - 111	62 - 79
16	73	6.64	CLAT_DROME	224 - 242	136 - 154
17	72	6.55	AAAA_ASPNI	177 - 192	90 - 105
18	72	6.55	AAAA_PENCH	177 - 192	90 - 105
19	72	6.55	CPT2_RAT	67 - 83	124 - 140
20	72	6.55	F13A_HUMAN	494 - 510	136 - 152

APPENDIX G

VOLUME 3

Study Title

Determination of Levels of Plant-Produced *Bacillus thuringiensis kurstaki* HD-1 Protein in Transgenic Maize

Authors

John A. Hanten

Ronald L. Meeusen

August 22, 1994

Performing Laboratory

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317 330th Street
Stanton, MN 55018

8 Pages

1 - NK5LVL

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this supplement on the basis of its falling within the scope of FIFRA 10(d)(A), (B) or (C).

Company: Northrup King Co.

Company Agent: Diana G. Williams

Diana G. Williams
Manager, Government Regulations

Date: August 22, 1994

This study does not meet the requirements of 40 CFR part 160.

Submitter & Sponsor: Northrup King Co.
Study Director: Ronald L. Meeusen, Ph.D.

Summary

Elisa analyses were conducted on leaf and seed tissues of transgenic maize lines to estimate the Btk HD-1 protein content in field trial lines. Results, corrected for the measured % extraction from each tissue, provide the following estimates of micrograms Btk HD-1 protein per gram fresh weight of plant tissue:

Btk HD-1 PROTEIN LEVELS			
MAIZE LINE	TISSUE	MEASURED (micrograms/g)	ESTIMATED (micrograms/g)
Bt 10	Mature Leaf	1.05	4.91[a]
	Young Leaf	1.06	3.75[b]
	Heterozygous Seed	1.68	4.44[c]
	Homozygous Seed	2.25	5.95[c]
Bt 11	Mature Leaf	3.26	15.23[a]
	Young Leaf	8.17	28.87[b]
	Heterozygous Seed	1.40	3.70[c]
	Homozygous Seed	1.80	4.76[c]

Extraction efficiencies from tissues were measured to be [a] 21.4% for mature leaf tissues, [b] 28.3% for young leaf tissues, and [c] 37.8% for seeds.

Materials and Methods

Tissue Samples:
Young leaf tissue was cut from six day old, greenhouse-grown corn seedlings. Mature leaf tissue was cut from the youngest fully expanded leaves of field-grown plants @ two weeks after pollen shed. Leaf tissues were immediately frozen and ground in liquid nitrogen with mortar and pestle. Dry seeds were ground in a waring blender.

Each leaf sample included 2 replicates of leaf tissue from 10 individual plants. Each seed sample included 10 seeds.

Extraction:
Subsamples (typically .5 g) of powders were weighed, added to 6 volumes of 50 mM Bis-Tris Propane pH 7.5, 5mM ethylene-diaminotetraacetic acid (EDTA), 5mM dithiothreitol (DTT) and mixed gently on ice for 20 minutes. Extracts were then centrifuged for 10 minutes at 3,000xg at 4°C. Supernatants were further clarified by centrifugation for 15 minutes at 11,000xg at 4°C. The clarified supernatants were used for Elisa analysis.

Elisa Analysis:
A sandwich assay was employed using a monoclonal antibody (provided by Monsanto) in conjunction with a rabbit polyclonal antibody raised against the purified microbial Btk HD-1 protein (HD-1t) provided by Monsanto, lot # I92017.

Microtiter plates were coated with a 1:4000 dilution of the monoclonal antibody in 60mM sodium carbonate/bicarbonate buffer, pH 9.6, and incubated overnight at 4°C.

After 3 five minute washes with PBST buffer (2.68mM KCL, 137mM NaCL, .07% Tween 20 and 10mM potassium phosphate, pH 7.4), test samples diluted in 0.2ml of PBSTO (PBST + 0.5% ovalbumen) were added to the wells. 0.05ml of a 1:500 dilution of the polyclonal antibody in PBSTO was then added, and the plates were incubated overnight at 4°C.

After 3 five minute PBST washes, .25ml of a 1:1500 dilution of an alkaline phosphatase-conjugated donkey anti-rabbit antibody in PBSTO was added and incubated for 1.5 hours at room temperature.

After 3 five minute PBST washes, color development was begun by adding .25ml of p-nitrophenyl phosphate in diethanolamine (20mg in 25ml). After one hour at room temperature absorbance at 620nm was read against standards (HD-1t provided by Monsanto, lot # I92017) on a SLT Lab Instruments (Austria) microtiter plate reader.

Results and Discussion

Northrup King Co. desires to conduct extensive field trials with maize lines, each carrying one of two separate insertion events (Bt10 and Bt11) of the same Btk HD-1 gene. Leaf and seed tissues from lines carrying each insertion were analyzed for HD-1 protein.

Leaf Tissue:
Elisa analyses were conducted on both young and mature leaf tissue of each transgenic line, Bt10 and Bt11, to determine whether levels are likely to change dramatically during the course of field trials. To estimate the % of Btk HD-1 protein extracted samples of non-transformed leaf tissue were prepared and spiked with 1 microgram of purified microbial Btk HD-1 protein (HD-1t sample provided by Monsanto, lot # I92017) per gram of frozen leaf powder:

Tissue Type	% Recovery
Mature Leaf	21.4% ± 2.3%
Young Leaf	28.3% ± 6.1%

These figures permit correction for the proportion of Btk HD-1 protein which remains unextracted in the tissues. The lower recovery from older leaf tissue is common, due to the tougher composition of mature leaves.

Maize lines containing the Bt11 insertion event produce higher levels of HD-1 protein than those with the Bt10 insert:

Btk HD-1 PROTEIN LEVELS			
MAIZE LINE	TISSUE	MEASURED (micrograms/g)	ESTIMATED (micrograms/g)
Bt 10	Mature Leaf	1.05 ± 0.17	4.91[a]
	Young Leaf	1.06 ± 0.59	3.75[b]
Bt 11	Mature Leaf	3.26 ± 0.67	15.23[a]
	Young Leaf	8.17 ± 1.95	28.87[b]

Extraction efficiencies from tissues were measured [a] 21.4% for mature leaf tissues, [b] 28.3% for young leaf tissues.

The results confirm that levels of HD-1 protein in transgenic maize leaf tissues are relatively constant, not changing significantly with age in lines with the Bt10 insert, and changing by less that 2x in lines with the Bt11 insert.

Seed Tissue:

Seed produced in field trials should also contain the HD-1 protein. Elisa analyses were therefore conducted on seed from transgenic maize lines with the Bt10 and Bt11 inserts. Open pollination in the field can produce three types of seed: seed lacking the Btk HD-1 gene, seed with one copy (heterozygous) of the gene, and seed with two copies (homozygous). To estimate the amounts of Btk HD-1 protein in seed from field tests it is necessary therefore to determine expression levels in both homozygous and heterozygous seed.

Extraction recovery from seed tissue was determined as described above for leaf tissue, and found to be 37.8% ± 8.3%.

Btk HD-1 PROTEIN LEVELS			
MAIZE LINE	TISSUE	MEASURED (micrograms/g)	ESTIMATED (micrograms/g)
Bt 10	Heterozygous Seed	1.68 ± 0.15	4.44
	Homozygous Seed	2.25 ± 0.30	5.95
Bt 11	Heterozygous Seed	1.40 ± 0.17	3.70
	Homozygous Seed	1.80 ± 0.37	4.76

Extraction efficiency from seed was measured to be 37.8%.

Little effect is evident from the state of homozygosity of the inserted Btk HD-1 gene in either of the transgenic maize lines. Estimation of Btk HD-1 levels in seed from field test plants, therefore, can readily be carried out by accounting for the 1:2:1 ratio of non-transformed, transformed heterozygous and transformed homozygous seed.

APPENDIX H

VOLUME 5

Study Title

Equivalence of Plant and Microbially Produced *Bacillus thuringiensis kurstaki* HD-1 Protein

Authors

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Date

August 22, 1994

43 Pages

1 - NK5EQ

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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Company: Northrup King Co.

Company Agent: Diana G. Williams
Diana G. Williams
Manager, Government Regulations

Date: August 22, 1994

This study does not meet the requirements of 40 CFR part 160.

Submitter & Sponsor: Northrup King Co.

Study Director: Ronald L. Meeusen, Ph.D.

Abstract

Northrup King Co. has developed insect resistant corn which expresses the gene for the parasporal crystal protein of *Bacillus thuringiensis var. kurstaki* (Btk). The safety of Btk parasporal crystal proteins has been demonstrated in over three decades of use as the active ingredient in Btk-based microbial insecticides. The specific gene used, CryIA(b), was isolated from Btk strain HD-1, the active ingredient in the most widely used Btk-based insecticides, Dipel.

Traditional safety studies require gram quantities of purified protein. However, purification of gram quantities from corn is not practical due to the very low levels of Btk HD-1 protein produced in corn. Therefore to assess the safety of the Btk HD-1 protein produced in corn *Escherichia coli* was used to produce 25 grams of the active, trypsin-resistant fragment of the Btk HD-1 protein for toxicity studies (detailed in Monsanto report # 11938).

To demonstrate that microbially-produced protein is an appropriate test material in place of the plant protein, studies were conducted to assess the equivalence of the two proteins.

The criteria and analytical methods used to establish equivalence are listed below:

Criterion	Analytical Technique
1. Apparent Molecular Weight:	SDS-PAGE and Western Blot
2. Immunological Equivalence:	Western Blot and ELISA
3. Trypsin Resistance:	Western Blot; SDS-PAGE Gel
4. Primary Amino Acid Sequence:	Amino-terminal Sequencing & Peptide Mapping of CNBr Fragments
5. Glycosylation of Protein:	DIG Glycan Analysis of Blotted Proteins
6. Bioactivity:	Insecticidal Activity in Diet Assays

Data from the above analyses show that the trypsin-resistant fragment of the Btk HD-1 protein, derived from a truncated form of the Btk HD-1 gene expressed in plants, is equivalent to the trypsin-resistant fragment of the full length Btk HD-1 protein produced in *E. coli*. Therefore, the microbially-produced protein was considered to be an appropriate test material for safety assessment studies in place of the plant-produced Btk HD-1 protein.

Table of Contents

List of Figures and Attachments

Purpose of the Study:

The purpose of this study was to evaluate the chemical and biological equivalence of plant-produced and microbially-produced Btk HD-1 protein.

Purification and trypsinization of the microbially-produced trypsin-resistant fragments of the HD-1 protein (HD-1t) were conducted by Monsanto company scientists, and documented by internal Monsanto company reports which are archived and held on file in the Monsanto company library. HD-1t was used as the test material for safety assessment studies, and samples of the same HD-1t lot were provided by Monsanto for these equivalence studies.

Purification and trypsinization of the plant-produced trypsin-resistant fragments of the HD-1 protein (HD-1tMz) were conducted by Northrup King Co. scientists, and documented by internal Northrup King Co. notebooks which are archived and held on file at the Stanton research facility.

Certain portions of the study were conducted under contract to the University of Wisconsin Biotechnology Center, and specific activities subcontracted to Kendrick Laboratories and the Washington University School of Medicine. Reports from this work are archived along with the Northrup King Co. internal records.

Safety assessment of the HD-1 protein supports regulatory approval of a truncated form of the HD-1 protein produced in corn. Since purification of large amounts of the HD-1 protein from plants is not practical, *Escherichia coli* expressing the Btk HD-1 parasporal crystal protein gene was used to produce 25 grams of purified HD-1t protein as test material for safety assessment studies. The studies conducted in this report demonstrate that the microbially-produced HD-1t protein was an appropriate test material to use in place of the plant-expressed HD-1 protein, as the maize protein is equivalent to the *E.coli* protein in all significant characteristics.

"Equivalence" Criteria:

The plant and microbial proteins were compared for chemical and biological equivalence using the following criteria and accompanying analytical methods:

Criteria	Analytical Techniques
1. Apparent Molecular Weight:	SDS-PAGE and Western Blot
2. Immunological Equivalence:	Western Blot and ELISA
3. Trypsin Resistance:	Western Blot; SDS-Page Gel
4. Primary Amino Acid Sequence:	Amino-terminal Sequencing & Peptide Mapping of CNBr Fragments
5. Glycosylation of Protein:	DIG Glycan Analysis of Blotted Proteins
6. Bioactivity:	Insecticidal Activity of Plant and Microbial HD-1 in Diet Assays

RATIONAL FOR SELECTING THE TRYPSIN-RESISTANT FRAGMENT OF THE B.t.k. HD-1 PROTEIN FOR SAFETY ASSESSMENT STUDIES:

As stated above, the purpose of the equivalence studies is to demonstrate the appropriateness of using microbially-produced HD-1 protein in place of plant-expressed HD-1 for safety assessment studies in mice and non-target insects. The criteria and analytical methods for demonstrating chemical and biological equivalence are outlined above. Equivalence studies for the HD-1 protein were performed with the trypsin-resistant fragment of the plant and microbially produced HD-1 proteins. The background and rationale for choosing this form of the protein is presented in detail below.

Background:

The natural insecticidal protein, HD-1, is produced in *Bacillus thuringiensis* var. *kurstaki* as a protoxin of approximately 130 kd (Hofte et al., 1989). After ingestion by sensitive lepidopteran insects, the protein is proteolytically cleaved in the alkaline conditions of the midgut to the activated form of the protein (Faust et al., 1967; Huber et al., 1981). *In vitro* proteolysis studies have shown that an insecticidally active fragment of

approximately 65 kd can be generated upon incubation with trypsin (Bietlot et al., 1989). This fragment consists of a sequence having an amino-terminus beginning at residue 29 and having a carboxy-terminus at or near amino acid 619. The 65 kd protein is called the trypsin-resistant fragment (HD-1t) of the HD-1 protein.

The genetically-modified, insect resistant corn lines, Bt10 and Bt11, contain a gene which codes for a protein of 615 amino acid residues (Attachment 1) identical in sequence to the first 615 residues of the full Btk microbial protein. Because of this sequence identity, the plant protein should, like the microbial protein, be subject to rapid proteolysis of the 28 residues at the amino terminus to liberate an equivalent @ 65 kd active fragment.

HD-1 protein extracted from corn tissue contains two related proteins in the expected size range: one of ~ 69 kd (the full 615 residues encoded by the gene total 68.9 kd) and one of ~ 65 kd protein (Figure 1), which would be expected if proteolysis had removed the first 28 amino acids. Some proteolysis is not unexpected since plants contain numerous proteases. Upon treatment with trypsin, the two related protein bands are converted to a single ~ 65 kd protein that is indistinguishable from the native HD-1t (see Figure 1).

Rationale:

There were several reasons for choosing the HD-1t instead of the truncated HD-1 protein for safety assessment studies. First, based on the information presented above, the HD-1t protein is common to the related HD-1 proteins observed in corn tissue extracts. Second, the truncated HD-1 protein was difficult to express at high levels in microbial systems. Both *E. coli* and *Bacillus thuringiensis* systems were evaluated with limited success. Third, the truncated HD-1 protein, like the full-length HD-1 protein, is rapidly converted to the trypsin-resistant fragment upon ingestion by the target insects; therefore, the HD-1t protein represents the true active ingredient. Fourth, it has been suggested that the protease-activated form (e.g. HD-1t) of the HD-1 protein may have a different insect host range than the full length HD-1 protein (Goldberg and Tjaden, 1990). The use of the trypsin-activated form (i.e. the HD-1t protein) is the most rigorous test of the safety of this protein.

Based on this rationale, the trypsin-resistant fragment from both the microbial and plant-expressed HD-1 proteins was generated and evaluated for chemical and biological equivalence. Equivalence with the plant HD-1t protein permits the microbial HD-1t protein to be used to assess the safety of the pure protein in mice and non-target insects.

Samples Used for Equivalence Studies:

HD-1t
The trypsin-resistant fragment of a microbially-produced HD-1 protein: Purified HD-1t protein derived from trypsinization of full-length HD-1 protein expressed in *E. coli*, lot # I92017. This HD-1t preparation was the actual test material evaluated for equivalence to the plant-produced protein. HD-1t was the material used for the mouse gavage study, and in-vitro digestability and non-target insect feeding studies conducted by Monsanto. This preparation contained three related HD-1 fragments comprising @ 90% of the total protein by SDS-PAGE analysis.

HD-1tMz
The trypsin-resistant fragment of the truncated HD-1 protein purified from leaf tissues of corn with the Btl1 gene insert: HD-1 protein produced in leaf tissue of transgenic maize expressing the Btk gene of pZO1502. Purification was effected by extraction of leaf tissue, trypsinization and immunoaffinity purification as described in the Methods section. From 675g of transgenic maize leaves approximately 0.9mg of HD-1tMz protein was isolated and used for the equivalency studies reported herein. This preparation contained three related Btk HD-1 fragments comprising @ 30% of the total protein by Elisa analysis.

Materials and Methods:

Extraction and Trypsinization of Maize HD-1 Protein:

Leaf tissue was cut from two week old greenhouse-grown Btl1 maize plants, frozen, ground to a fine powder in liquid nitrogen using a Waring blender, and stored at -80°C.

Extraction buffer was prepared by mixing 0.25kg of polyvinylpolypyrrolidone (PVPP) prepared according to Loomis (Loomis, 1974; Loomis et al., 1979) into each 6 liters of 50mM Bis-Tris Propane (pH 7.5), 5mM ethylenediaminotetraacetic acid (EDTA), 5mM dithiothreitol (DTT) and letting the slurry stand at 4°C for two hours. Immediately before adding the maize powder, phenyl methylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1mM. 6.25 ml of extraction slurry was used per gram of maize powder.

Maize leaf powder was weighed out while frozen, added to the PVPP/buffer slurry, stirred until the frozen powder chunks thawed, and then for another 5 minutes. All subsequent steps were carried out at 2-4°C. The mix was filtered through two layers of Miracloth (Calbiochem) and centrifuged for 20 minutes at 17,000xg. The supernatant was removed and ammonium sulfate added to 1.0M. The supernatant was then stirred for 15 minutes, allowed to stand another 45 minutes, and centrifuged at 17,000xg for 45 minutes. The supernatant was again removed, ammonium sulfate added to 1.6M, and again stirred, allowed to stand and centrifuged as before. The pellet of this 1.6M ammonium sulfate precipitation was resuspended in 1/20 the original extraction volume of 50mM Bis-Tris Propane (pH 9.0).

After determination of protein content with the Bio-Rad Bradford assay, one mg of trypsin (Sigma Type VIII) was added for every 30 mg of protein, and the solution stirred for 12 hours. 0.1M PMSF in propanol was then added to 0.1mM to stop the trypsin reaction, the solution was centrifuged at 48,000xg for 20 minutes and the pellet discarded.

Immunopurification of HD-1tMz Protein:

Rabbit polyclonal antibodies were raised against the microbially-produced, trypsinized HD-1 protein used in safety assessment studies (HD-1t Lot # I92017). 358 mg of IgG antibody was incubated with 15 g of CNBR-activated Sepharose 4B (Sigma Chemical Co.) using the manufacturer's protocol (Pharmacia publication 71-7086-00). After coupling and washing, a 20ml bed volume column was prepared and equilibrated with 10mM potassium phosphate, pH 7.2.

Protein extracted from transgenic maize tissue (the resolubilized 1.0 to 1.6M ammonium sulfate fraction) was loaded onto the column in a single pass. The column was then washed with 35ml of 10mM potassium phosphate (ph 7.2), 18 ml of 100mM potassium phosphate (pH 9.7) and another 13 ml of 10mM potassium phosphate (ph 7.2).

HD-1tMz was eluted with 44 ml of 100mM potassium phosphate (pH 11.8). After washing with 15ml of 10mM potassium phosphate (pH 7.2), 12 ml of 100mM glycine buffer (pH 2.5) was used to elute more HD-1tMz. The base and acid elutions were neutralized, combined and precipitated a final time with 1.6M ammonium sulfate to concentrate the protein. The ammonium sulfate pellet was resuspended in 2.4ml of 100mM sodium carbonate buffer, pH 10.0 (the same buffer used for the HD-1t).

SDS-PAGE: SDS slab gel electrophoresis was carried out according to the method of Laemmli (1970) as modified by O'Farrell (1975) using a 10% acrylamide slab gel and 5% acrylamide stacking gel. Gels were cast and run in a Hoefer Mighty Small apparatus. Glycosylation studies employed a larger, higher resolution (125mm length x 150mm width x 1.5mm thickness, 25 mm stacking gel) slab gel.

Coomassie Staining: Gels were fixed in Coomassie Buffer (10% acetic acid, 30% ethanol) for 10 minutes, stained in 0.1% Coomassie Brilliant Blue in Coomassie Buffer for 15-20 minutes, and destained in 3-4 rinses of Coomassie Buffer.

Western Blotting: Proteins were transferred to nitrocellulose membranes from acrylamide gels in a Hoefer TE70 semi-dry transfer unit using Towbin Transfer Buffer (25mM Tris-HCL, 192mM Glycine, 1.3mM SDS, 15% methanol pH 8.3)and a current of 38 milliamperes for 45 minutes. The membranes were blocked overnight at 4°C with 1% bovine serum albumen (BSA) in TBST Buffer (150mM NaCl, 0.05% Tween 20, 10mM Tris-HCL pH 8.0), washed 3 times for 10 minutes each with TBST Buffer, and incubated for 45 minutes with the primary antibody diluted 1:1000 in TBST. The membranes were then washed 3 times for 10 minutes each in TBST, incubated 45 minutes with phosphatase labeled goat anti-rabbit IgG diluted 1:7500 in TBST, and washed 3 times for 10 minutes each in TBST. The membranes were blotted dry on Whatman #1 filter paper, developed with Western Blue stabilized substrate for alkaline phosphatase (Promega) while gently shaking, washed with water and allowed to dry for darker color development.

N-terminal Amino Acid Sequencing: Protein samples were blotted from SDS-PAGE gels onto PVDF membranes and sequenced on an Applied Biosystems model 470A sequencer with an "on-line" model 120A PTH analyzer. Standard manufacturer reagents and cycles were employed. The PTH-AA data was collected both on stripchart and into the ABI 610 software.

Glycosylation Analysis:

Samples were diluted in 5% sodium dodecyl sulfate (SDS), 10% glycerol, 5% beta-mercaptoethanol and 63mM Tris buffer at pH 6.8. After electrophoresis on a 10% Laemmli slab gel (10) as modified by O'Farrell (13) the gel was cut in half and lanes 3-7 blotted onto a PVDF membrane for labeling for glycosylated proteins using the DIG Glycan/Protein Double Labeling Kit from Boehringer Mannheim. Lanes 10-14 were stained for total protein with Coomassie Brilliant Blue stain.

Peptide Mapping:

Indicated amounts of samples were treated with 200 microliters of 50mg/ml cyanogen bromide (CNBR) in 70% formic acid overnight and dried repeatedly under nitrogen to remove the formic acid. Dried samples were dissolved in SDS buffer (as in glycosylation above) and heated to 95°C for 2 minutes. Samples were loaded onto a 16.5% acrylamide peptide slab gel (Shaggers and Jagow, 1987) Electrophoresis was carried out at 15 milliamp/gel for four hours, then at 10 milliamp/gel overnight, and the gel blotted onto PVDF membrane before staining with Coomassie Brilliant Blue.

ELISA Protocol:

Maize extract, HD-1t or purified HD-1tMz were serially diluted with Coating Buffer (100mM Sodium Carbonate, pH 9.6) in 96 well microtiter plates, and 0.05ml added to each well. Plates were sealed with parafilm and incubated overnight at 4°C. Coating buffer was removed and the wells blocked with 0.2ml of Diluting Buffer (0.05% Tween 20, 0.25% BSA PBS: 3.4 mM KCl, 170mM NaCl and 9.9mM sodium phosphate buffer, pH 7.4) for one hour at room temperature.

The wells were rinsed twice with Wash Buffer (0.05% Tween 20 in PBS), incubated one hour at 37°C with 0.05ml/well of primary antibody diluted 1:1500 in Diluting Buffer, and washed three times with Wash Buffer. Wells were then incubated one hour at 37 C with 0.05ml/well of alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:2000 in Diluting Buffer, washed three times with Wash Buffer, and washed twice with 10mM diethanolamine-0.5mM $MgCl_2$. To each well 0.05ml of Substrate Solution (1mg/ml p-nitrophenyl phosphate in 10mM diethanolamine-0.5mM $MgCl_2$) was added and the color allowed to develop for 15 to 30 minutes. The reaction was stopped with 0.05ml/well of 0.1M EDTA and absorbance at 405nm read.

Quantitation of Gels:

Coomassie gels were scanned at 100 microns resolution using a LGS-50 Laser Scanning Densitometer (Digital Instruments Corp., Newark, DE). The scanner was checked for linearity prior to scanning with a Melles Griot Neutral Density Filters Set (Melles Griot, Irvine, CA).

Western Blots were scanned on a HP Scanjet IIp Scanner at a resolution of 88 microns. Scanner Response was linearized as described by Kendrick et al.(1984).

Background level was set at two standard deviations above the mode value, or at a value that subtracted away noise while not removing the protein bands.

Insect Bioassays: Artificial diet for European Corn Borer (*Ostrinia nubilalis, Hubner*) was prepared according to Guthrie et al (1985), excluding formaldehyde and substituting cholesterol for beta-sitosterol. Test proteins (HD-1t or HD-1tMz) were added to aliquots of the diet while still liquid (@ 45°C) and mixed. 0.3ml of diet was then dispensed into each 1.6cm dia x 1.6cm deep well. After diet had solidified three neonate larvae were added to each well. ECB mortality and weights were determined after 7 days on diet.

Artificial diet for Corn Earworm (*Helicoverpa zea*, Boddie) was prepared according to King and Hartley (1985). Test proteins were added and diets dispensed as described above. One larva was added per well to avoid cannibalism, and weights taken after 5 days.

Results and Discussion:

Purification of HD-1tMz from Transgenic Maize

To obtain purified HD-1 protein from transgenic maize, frozen leaf tissue was ground in liquid nitrogen, and soluble protein was extracted (Figures 1a & 1b, lanes 2). The extract was further purified by ammonium sulfate precipitation (lanes 3), and the HD-1 containing fraction was then treated with trypsin to convert the protein to its trypsin-resistant core fragment. This step also enhanced purification by degrading non-trypsin resistant proteins (lanes 4). Finally the HD-1 (now named HD-1tMz) protein was isolated by immunopurification (lanes 5).

Figures 1a and 1b show Western Blot and SDS-PAGE analyses of fractions during the purification of Btk protein from transgenic maize leaf tissue. As expected, total soluble protein from transgenic maize contains a protein which reacts with antibodies raised against microbial Btk protein (the test material used for toxicity studies). The HD-1 reactive material produced in the plant (Figure 1a, lanes 2 & 3) is primarily composed of a doublet in the 65-69 kd range, and a small amount of two lower molecular weight materials at @ 40 and @ 15 kd.

Trypsinization converts the 65-69 kd doublet to a single @ 65 kd band (lanes 3 & 4) of the same apparent molecular weight as the microbially-produced, trypsinized HD-1t[1] test sample (lane 6). In both cases the active "core" fragment of @ 65 kd is generated, which has been identified previously to be the biologically active form of the native Btk HD-1 protein (1,2,4).

The Btk gene inserted into maize codes for a protein of 615 amino acids (predicted MW 68,910), with a trypsin sensitive site after amino acid # 28 (Figure 10). Cleavage by trypsin is therefore predicted to reduce the molecular weight of the core fragment by 3,134 daltons, to 65,776. The presence of a doublet in the 65 to 69 kd range in the plant extract (Figure 1a, lanes 2 & 3) is consistent with the plant producing the expected @ 69 kd protein, followed by some proteolysis at the trypsin sensitive site.

Some lower molecular weight immunoreactive materials, of @ 42 and @ 15 kd, are present in HD-1tMz preparations, indicating presence of some partially degraded Btk protein as well. Similar degradation products are also observed in the standard HD-1t test material. These will be discussed in more detail in section 4 of the Equivalence Studies.

The purity of the HD-1tMz preparation can be estimated by quantitating on a Coomassie stained gel the three bands (65, 40 and 15 kd, Figure 1b) which react with antibodies to authentic HD-1 protein in Western Blots (Figure 1a). Scanning of the Coomassie gel (Figure 1b) with a LGS-50 Densitometer indicates these three bands account for @ 80% of the stained protein in the HD-1tMz preparation. A more accurate quantitation by Elisa indicates @ 30% of the total protein is HD-1 protein.

675g of powdered maize leaf tissue extracted in 4.2 liters of slurry yielded @ 0.9mg of HD-1tMz.

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[1]This microbially-produced Btk HD-1 sample used for toxicity testing in mice had been similarly converted to the active -65 kd core form by trypsin treatment prior to use (Lot # I92017 Monsanto Report).

Equivalence Studies with Plant and Microbially-Produced HD-1 Proteins

1. Equivalence of Apparent Molecular Weight

SDS-PAGE and Western Immunoblot analyses compared the apparent molecular weight of the transgenic maize Btk preparation (HD-1tMz) to that of the test material used in toxicity studies (HD-1t). The Western Blot in Figure 2a, and companion Coomassie stained SDS-PAGE gel in Figure 2b, show that the predominant protein bands in both preparations are equivalent in apparent molecular weight (@65 kd). This is the expected molecular weight of the biologically active core fragment of Btk HD-1 protein, and the predicted trypsinized product of the inserted Btk HD-1 gene (Attachment 1).

In addition to the active @65 kd core fragment Figures 2a and 2b also show smaller amounts of proteolytic degradation products. Both preparations produce two major breakdown products, two of which appear of equivalent molecular weight (@ 40kd). The other two differ, which is not unexpected considering differences in source organism and purification methods. (Refer to section 4 for details).

Therefore HD-1t and HD-1tMz contain core Btk HD-1 fragments of equivalent apparent molecular weight (@ 65 kd) as judged by both Western Blot and SDS-PAGE analyses.

2 Immunological Equivalence

The Western analyses above (Figures 1a and 2a) demonstrate that the maize HD-1tMz @ 65kd core fragment, and its major degradation products, are recognized by antibodies to the microbial Btk HD-1t protein. Similar amounts of HD-1tMz and HD-1t stain with similar intensities.

Elisa dose responses provide a more quantitative comparison of antigen/antibody interactions. Figure 3 compares the dose responses of maize HD-1tMz and microbial HD-1t proteins. Two proteins with the same primary amino acid sequence would be expected to produce similar dose response curves, unless one folded anomolously, significantly changing the epitopes available for antibody binding.

The similarity of the HD-1tMz and HD-1t dose response curves suggests that these two proteins are immunologically equivalent.

3 Equivalence of Trypsin Stability

The resistance of the Btk HD-1 protein's core fragment to trypsin is an unusual property among proteins. The core fragment contains 43 internal residues (arginines and lysines) at which trypsin could cleave. It is commonly accepted that the conformation of the core fragment buries these residues tightly within the three dimensional structure so that trypsin does not reach them.

If the three dimensional conformation of the plant-produced core fragment differed significantly from that of the microbial protein one might expect one or more of these 43 trypsin sensitive residues to be uncovered, reducing the fragment's trypsin resistance. That the core fragment of the plant produced protein, HD-1tMz, showed no visible degradation by trypsin after 12 hours of trypsinization (Figure 1a, lanes 3 & 4) strongly suggests that HD-1tMz, like HD-1t, is tightly folded so as to protect its many sensitive residues from trypsin.

4 Equivalence of Primary Amino Acid Sequences

N-Terminal Sequence Analysis

N-terminal amino acid sequencing by Edmund degradation was carried out on protein blotted from the major SDS-PAGE bands of the maize Btk protein, HD-1tMz, as well as the HD-1 test material. Results confirm that the HD-1tMz core ~65 kd fragment possesses the amino terminal sequence predicted:

	21	31	41
Predicted:	..VEVLGGER[a]	IE TGYTPIDISL	SLTQF
HD-1tMz 65kd Result		XE XXYXPIDISL	SLTQF
HD-1 65kd Result		XE TG...	

[a]Trypsin Cleavage Site

(X Represents unassignable signal from sequencer.)

The predicted sequence of the @ 65 kd core fragment extends from amino acid #29 to #615. Results of N-terminal sequencing shown above confirm that the maize @ 65 kd core fragment has the amino terminal sequence expected for an authentic HD-1 protein after trypsinization. This also confirms that HD-1tMz has, as expected, the same amino terminal sequence as the HD-1t test material.

N-terminal amino acid sequencing also confirmed that the immunoreactive, lower molecular weight materials in HD-1tMz and HD-1t are degradation products of the core fragments. The @ 40 kd bands in HD-1Mz and HD-1t start within two amino acids of the original trypsinized core fragment:

```
                  21          31          41
Core:        ..VEVLGGER IE TGYTPIDISL SLTQF
Maize 40 kd Result:        TGYTPIDISL SLTQF
```

The smaller HD-1tMz band at @ 15 kd is shown to be a fragment from the carboxy end of the core fragment, starting at amino acid # 443:

```
                          441         451         461
Core:                  ...SNSSVSIIRA PMFSWIHRSA EFN...
Maize 15 kd Result:        XXVSIIRA PMF....
```

Equivalence of Peptide Fragments

Proteins with equivalent amino acid sequences would be expected to produce highly similar peptides on digestion with cyanogen bromide (CNBR). Figure 4 shows a comparison of HD-1tMz and HD-1t after CNBR digestion, demonstrating the predicted equivalence.

5 Equivalence of Protein Glycosylation

Proteins produced in plants are typically not glycosylated unless targeted to the endoplasmic reticulum. Even though the Btk protein in maize was not targeted to the endoplasmic reticulum, studies were conducted on the maize Btk protein to confirm that the plant-produced and microbially-produced proteins do not differ in this respect.

Both protein samples, HD-1t and HD-1tMz, were run out on an SDS-PAGE gel and blotted onto a polyvinyl difluoride (PVDF) membrane. The membrane was then stained for carbohydrate using the DIG/Glycan double labeling method (Boehringer Mannheim). This method labels both proteins and glycoproteins transblotted onto a PVDF membrane and visualizes them in different colors. Glycosylated proteins stain blue, and non-glycosylated proteins reddish-brown.

Figure 5a shows the results after staining for glycoproteins. Neither the microbially-produced (HD-1) nor the maize-produced (HD-1tMz) proteins stained blue even at 7 times the loading which gave strong staining of the positive control (Fetuin). Presence of both Btk samples on the blot is confirmed by subsequent Coomassie staining of the other half of the gel (Figure 5b).

As expected, these results demonstrate that no significant post-translational glycosylation of the Btk HD-1 protein has occurred in maize.

6. Equivalence of Bioactivity

The HD-1 protein of Btk is specifically toxic to Lepidopteran larvae, reducing weight gain when ingested at low levels and killing the insect pest at higher doses. Diet-incorporation feeding studies are the standard assay for Btk protein bioactivity.

The plant-produced protein (HD-1tMz) was compared to the microbial protein (Hd-1t) in diet incorporation assays with two Lepidopteran insect pests of maize. The European Corn Borer (ECB or *Ostrinia nubilalis*) is the primary Lepidopteran pest of maize in the United States. Corn Earworm (CEW or *Helicoverpa zea*) is a second major US corn pest.

European Corn Borer: Figure 6a presents the mortality response of ECB to increasing doses of HD-1t and HD-1tMz in artificial diet. Both the shapes and ranges of the two dose-response curves appear equivalent. Plotted as a log dose response (Figure 6b), it is clear that the doses chosen for this assay effectively covered the full response range of the insect to both HD-1t and HD-1tMz, with good coverage of the important central range of the curves.

Probit transformation and regression analysis using the log dose values as the dependent variable (Figure 6c) confirms that the slopes of the mortality response lines for HD-1t and HD-1tMz are equivalent (0.503 vs 0.513 respectively). High R^2 values of .977 and .964 confirm the robustness of the data and analysis. From these probit regression lines a comparison of the LD_{50} (the dose of HD-1 which resulted in 50% mortality) values can be determined:

	LD_{50} (µg/ml)	95% Confidence
Plant Protein (HD-1tMz)	0.47	0.33 - 0.66
Microbial Protein (HD-1t)	0.50	0.38 - 0.66

Figure 7a compares the effects of HD-1tMz and HD-1t on weight gain by European Corn Borer larvae. As with mortality, both proteins produce very similar effects at similar levels. Plotted as log weight vs log dose (Figure 7b) it is again clear that the doses chosen for this assay effectively covered the full response range of the insect.

Probit transformation of the weight data, expressed as a percent of control and calculated using log dose as the dependent variable (Figure 7c) provided a linear relationship for estimation of the EC_{50} (the dose which reduces weight gain by 50%):

	EC_{50} (µg/ml)	95% Confidence
Plant Protein (HD-1tMz)	0.060	0.046 - 0.076
Microbial Protein (HD-1t)	0.067	0.042 - 0.107

The plant-produced protein, HD-1tMz, is equivalent to the test material, HD-1t, in its biological activity against the European Corn Borer as measured by mortality at higher doses and weight reduction at lower doses.

Corn Earworm: Figure 8a presents the affects of HD-1tMz and HD-1t on CEW weight gain. Dose response curves for HD-1tMz and HD-1t proved very similar in this organism as well, reducing weight over the same range of doses with response curves of similar shape.

The similarity in biological responses is more apparent when the data is plotted as log weight vs log dose (Figure 8b). (The limited amount of HD-1tMz available from maize tissues precluded use of the higher doses necessary to conduct mortality assays in this less sensitive insect.)

Summary of Bioactivity Data:

Since the biological activity of a protein is a function of its primary amino acid sequence and conformation, the insecticidal activity of Btk HD-1 proteins may be considered the most significant test of equivalence.

BIOLOGICAL ACTIVITY vs Ostrinia nubilalis		
Parameter	HD-1t	HD-1tMz
LD 50	0.50 (0.38-0.66	0.47 (0.33-0.66)
Slope of Regression Line for Mortality	0.503 ± 0.034	0.513 ± 0.045
R^2 Value for Regression Line	.977	.964
Intercept	-2.82 ± 0.17	-2.89 ± 0.24
EC 50	0.067 (0.042-0.107)	0.060 (0.046-0.076)
Slope of Regression Line for Weight Loss	-0.389 ± 0.039	-0.367 ± 0.022
R^2 Value for Regression Line	.980	.993
Intercept	0.772 ± 0.170	0.612 ± 0.102

HD-1tMz and HD-1t produce mortality and weight reduction effects over the same ranges and response curves of similar shape, as would be expected of proteins with equivalent potencies and mechanisms of action. The LD_{50} and ED_{50} values of the plant generated HD-1 protein and the slopes of regression lines for the respective response curves proved to be equivalent to those of the microbially generated HD-1 protein used as the test material for toxicity studies. Thus the biological activities of the two samples are considered to be equivalent.

Summary and Conclusions:

The following table summarizes the analyses conducted to test equivalence of the two HD-1 proteins:

SUMMARY OF EQUIVALENCE STUDIES: HD-1t and HD-1tMz		
CRITERION	**TEST SYSTEM**	**RESULT**
Molecular Weight	SDS-PAGE Western Blot	Same Apparent MW
Immunological Equivalence	Western Blot	HD-1t Antibody Reacts to HD-1tMz
	HD-1 Elisa	Dose-Response Curves Equivalent
Conformation	Trypsin Digestion	Equivalent Resistance
Amino Acid Sequence	N-Terminal Sequencing	N-Terminal Residues Match Prediction.
	Peptide Mapping	HD-1tMz and HD-1t Fragments Equivalent.
Glycosylation	Carbohydrate Staining of Blotted Proteins	No Carbohydrate Detected in HD-1tMz or HD-1t.
Bioactivity	Insecticidal Activity in Feeding Assays	Plant and Microbial HD-1's Display Equivalent Bioactivities

Data from the above analyses show that the trypsin-resistant fragment of the Btk HD-1 protein, derived from a truncated form of the Btk HD-1 gene expressed in plants, is equivalent to the trypsin-resistant fragment of the full length Btk HD-1 protein produced in *E. coli*. Therefore, the microbially-produced protein was considered to be an appropriate test material for safety assessment studies in place of the plant-produced Btk HD-1 protein.

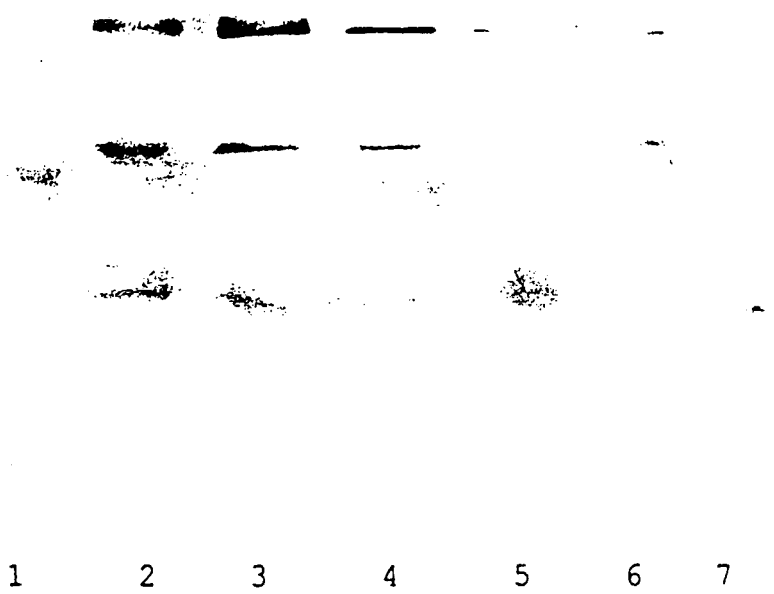


Figure 1a: Purification of HD-1tMz from Transgenic Maize - Western Blot. Sequential samples from the purification of HD-1tMz from transgenic maize leaves were run on SDS-PAGE, blotted onto nitrocellulose and visualized with rabbit anti HD-1t serum. Lanes 1 and 7 = Molecular weight markers. Lane 2 = 5µl Crude extract of total leaf soluble protein. Lanes 3&4 = 5µl of the 1.0-1.6M ammonium sulfate fraction before and after trypsinization. Lane 5 = 10ng of final, immunopurified HD-1tMz. Lane 6 = 10ng of HD-1t standard.

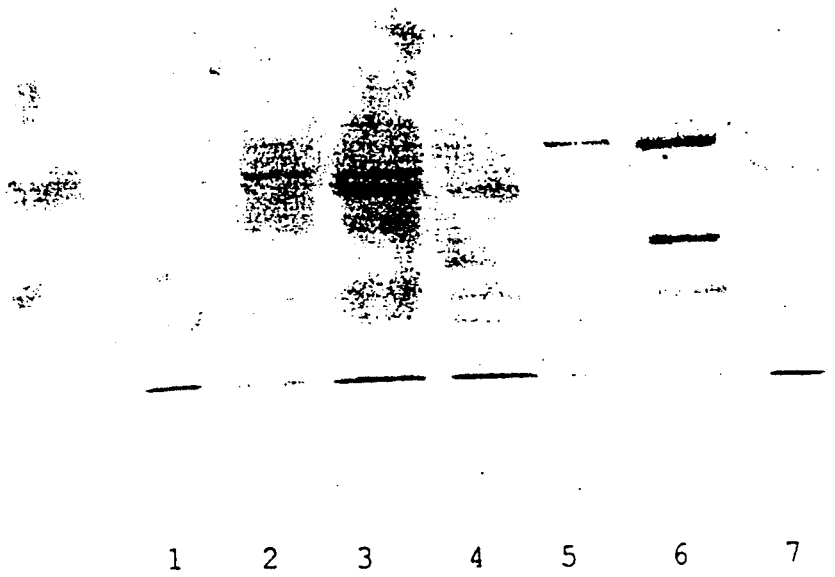
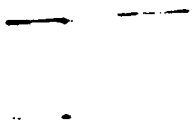


Figure 1b: Purification of HD-1tMz from Transgenic Maize; SDS-PAGE: Sequential samples from the purification of HD-1tMz from transgenic maize leaves run on SDS-PAGE and stained with Coomassie Brilliant Blue. (Same samples as Figure 1a) Lanes 1 and 7 = Molecular weight markers. Lane 2 = 5µl Crude extract of total leaf soluble protein. Lanes 3&4 = 5µl of the 1.0-1.6M ammonium sulfate fraction before and after trypsinization. Lane 5 = 5µg of final, immunopurified HD-1tMz. Lane 6 = 5µg of HD-1t standard.



1 2 3 4 5 6 7 8

Figure 2a: Western Blot Comparison of Plant (HD-1tMz) and Microbial (HD-1t) Proteins: Lanes 1 & 8 = Molecular weight markers. Lanes 2, 4 & 6 = 5ng, 10ng and 20ng (total protein) of HD-1t from *E. coli*. Lanes 3, 5 & 7 = 5ng. 10ng & 20ng (total protein) of HD-1tMz from Maize.

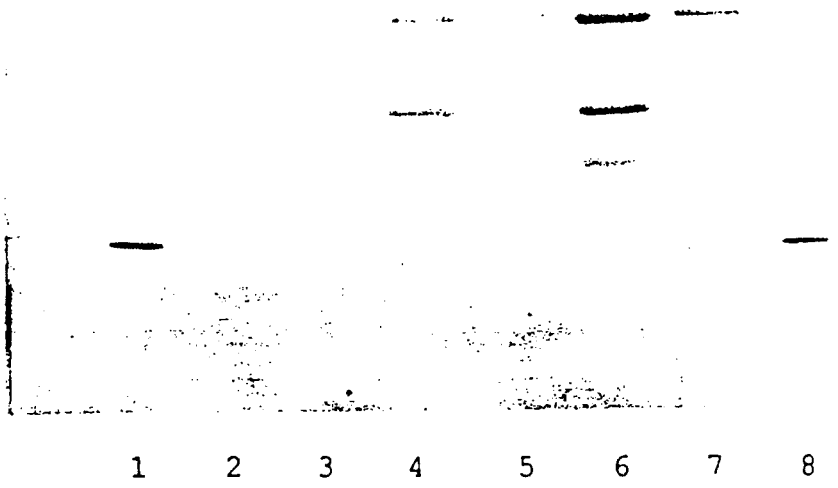


Figure 2b: SDS-PAGE Comparison of Plant (HD-1tMz) and Microbial (HD-1t) Proteins: Lanes 1 & 8 = Molecular weight markers. Lanes 2, 4 & 6 = .25µg, 2µg and 5µg (total protein) of HD-1t from *E. coli*. Lanes 3, 5 & 7 = .25µg, 2µg & 5µg (total protein) of HD-1tMz from Maize.

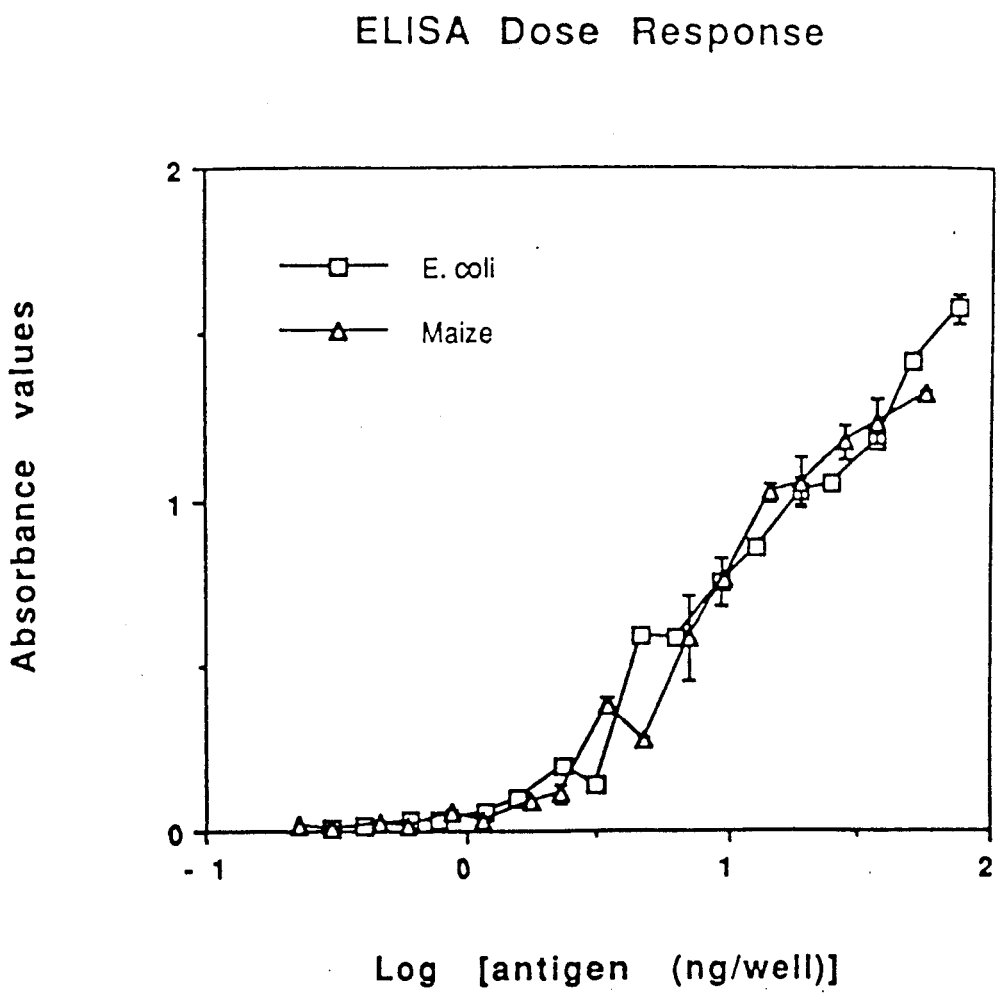


Figure 3: Comparison of Elisa Dose Responses of HD-1tMz and HD-1t. Data points represent means of duplicate samples. Bars represent standard errors.

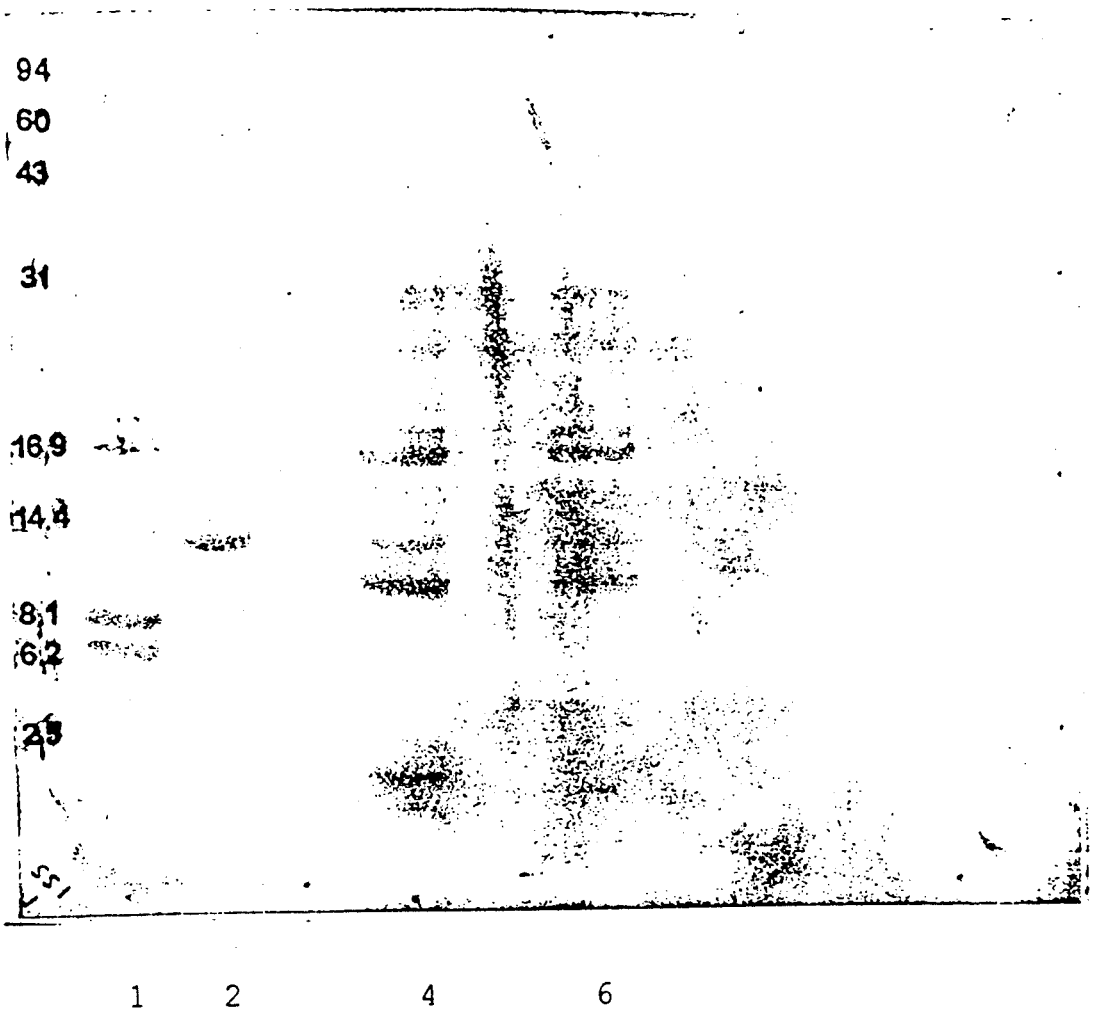


Figure 4: Peptide Map of CNBr Digested HD-1tMz and HD-1t. 50 micrograms of HD-1tMz (lane 6) and 30 micrograms HD-1t (lane 4) were partially digested with CNBr, run on a 16.5% polyacrylamide gel and blotted onto a PVDF membrane. All prominent bands are present in both samples. No significant differences are apparent from this analysis.

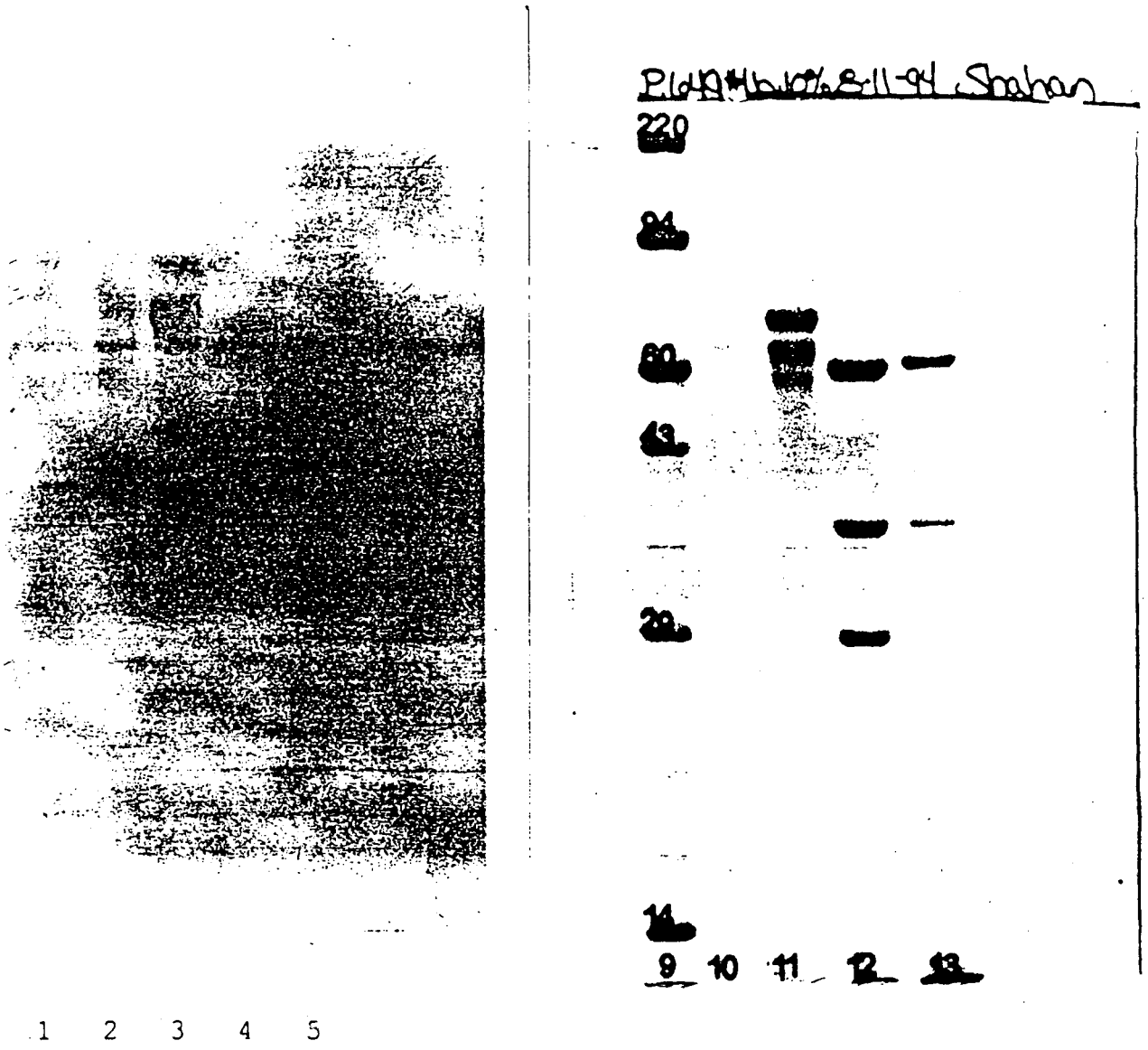


Figure 5: Glycosylation Analysis of HD-1tMz and HD-1t. (a) Blotted membrane stained for carbohydrate (blue). Lane 1 = Molecular weight standards. Lanes 2 & 3 = 1 & 7µg of Fetuin (Glycosylated Control). Lane 4 = 7µg of HD-1t. Lane 5 = 7µg of HD-1tMz. (b) Coomassie stained SDS-PAGE gel of samples in (a). Lane 9 = Molecular weight standards. Lanes 10 & 11 = 1 & 7µg of Fetuin (Glycosylated Control). Lane 12 = 7µg of HD-1t. Lane 13 = 7µg of HD-1tMz.

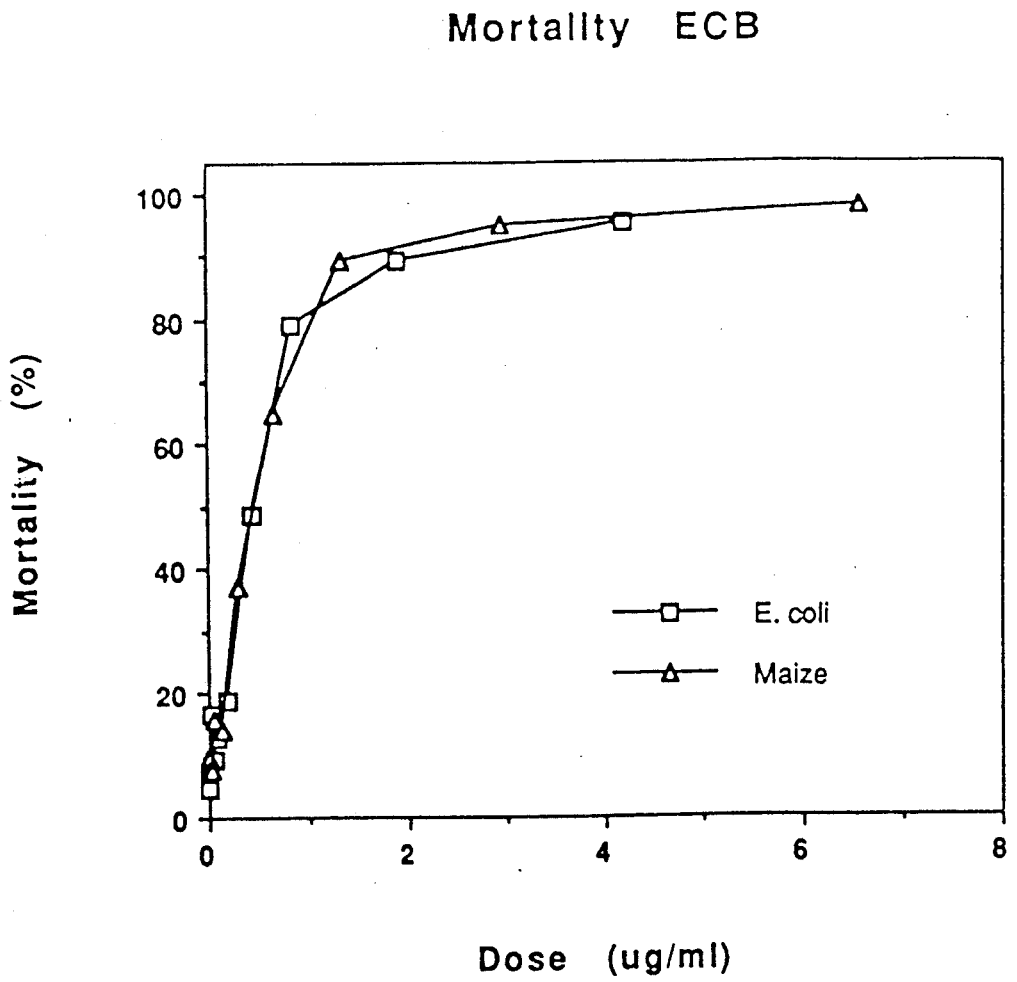


Figure 6a: European Corn Borer Sensitivity to HD-1tMz and HD-1t - Mortality. Mortality was scored after 6 days on artificial media treated with test proteins. Each data point represents % mortality from 35 to 45 insects.

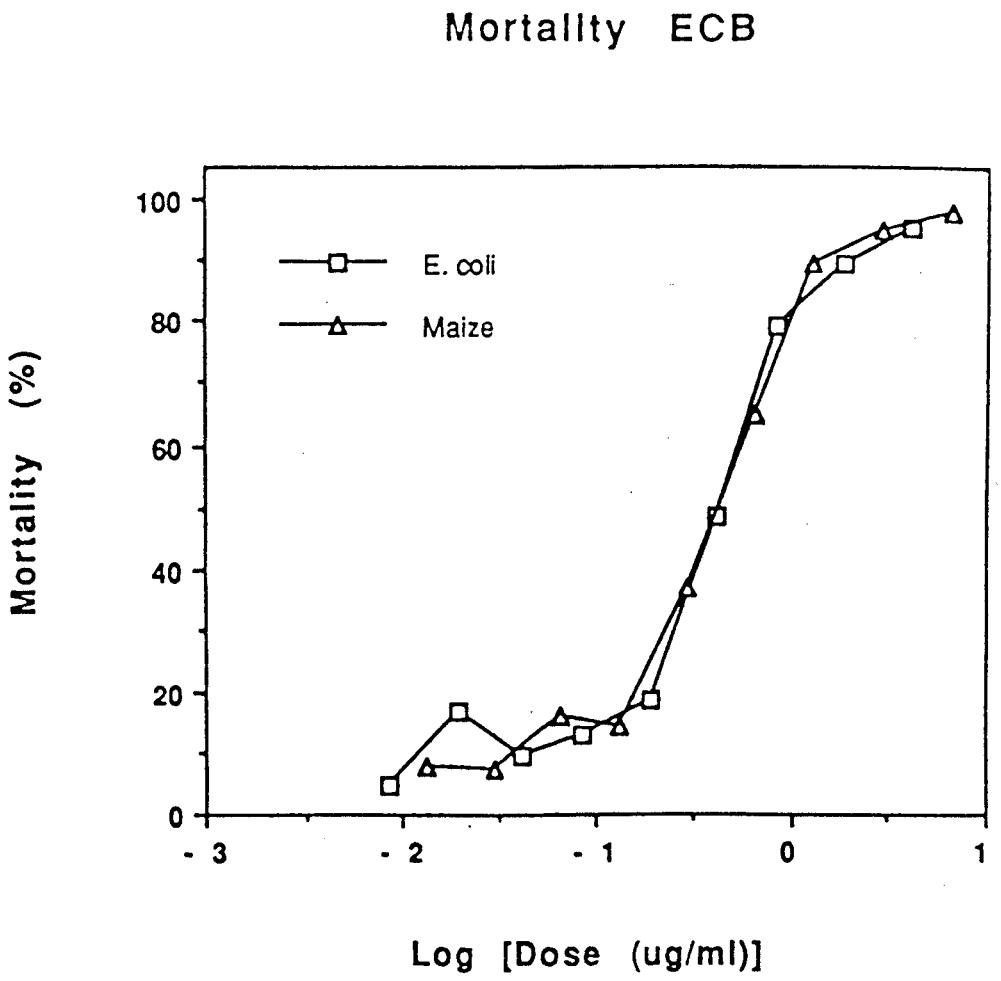


Figure 6b: European Corn Borer Sensitivity to HD-1tMz and HD-1t - Mortality. Data from figure 6a expressed as log of dose.

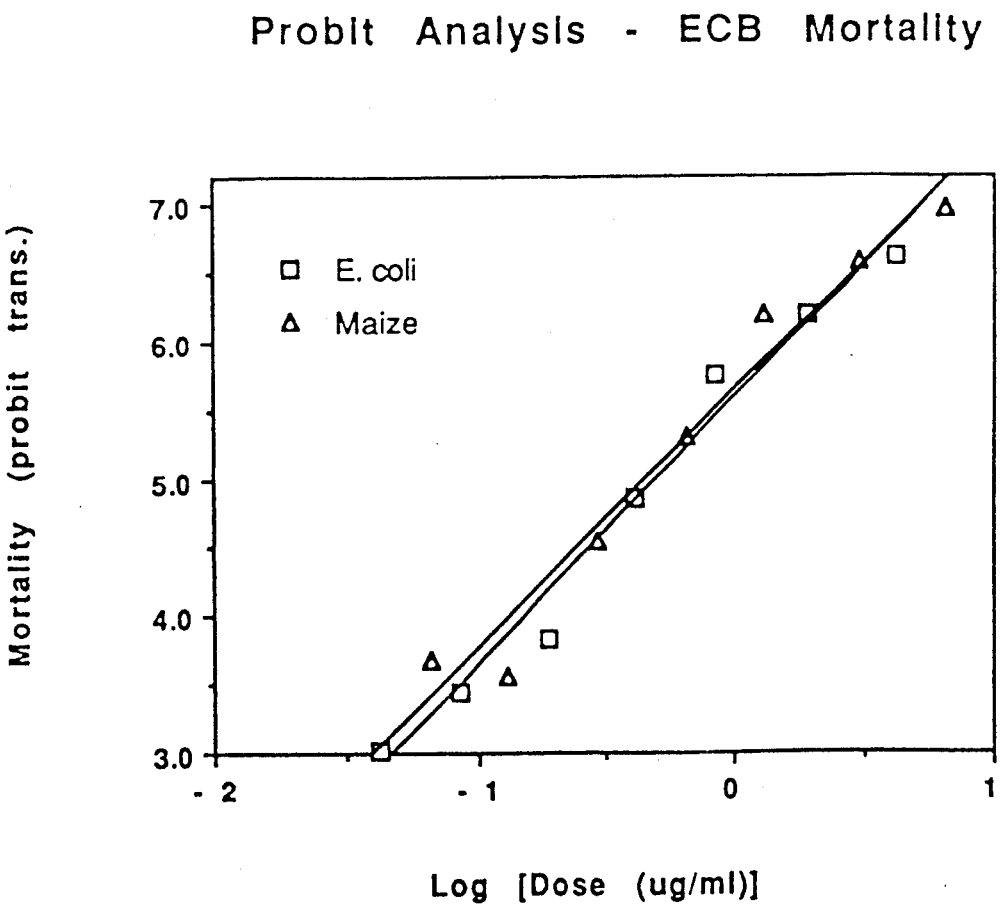


Figure 6c: Probit Analysis of European Corn Borer Sensitivity to HD-1tMz and HD-1t - Mortality. Mortality data from figure 6a was transformed using Probit tables for adjusted % mortality (P = [P'-C]/[1-C]) and expressed against the log dose. Lines represent regression analyses for each protein's effect. The LD_{50} values reported in the text were calculated from these regression lines.

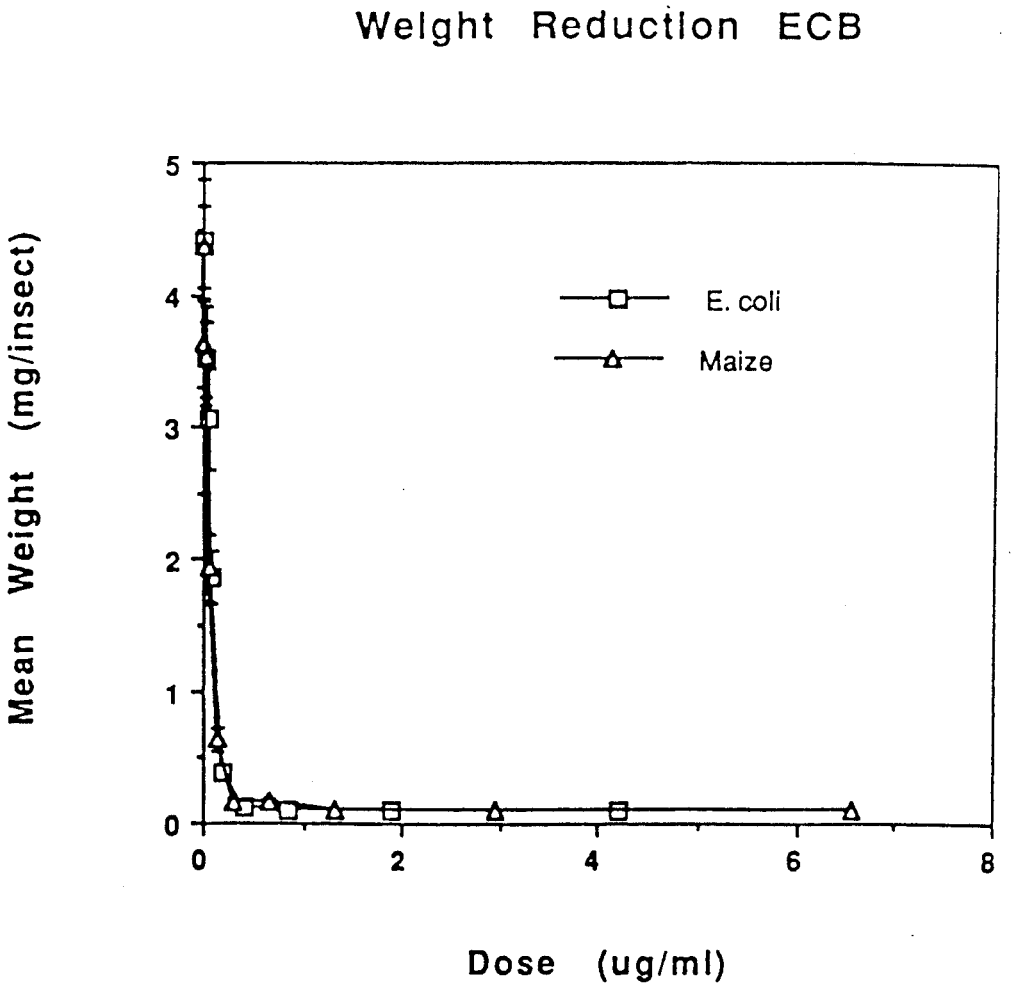


Figure 7a: European Corn Borer Sensitivity to HD-1tMz and HD-1t - Weight. Weights were taken of the surviving insects from the test presented in Figure 6. Survivors from each well (3 insects/well) were weighed together after 7 days. Each data point represents mean weight of the survivors from the initial 35 to 45 insects treated at each dose. Bars represent standard errors.

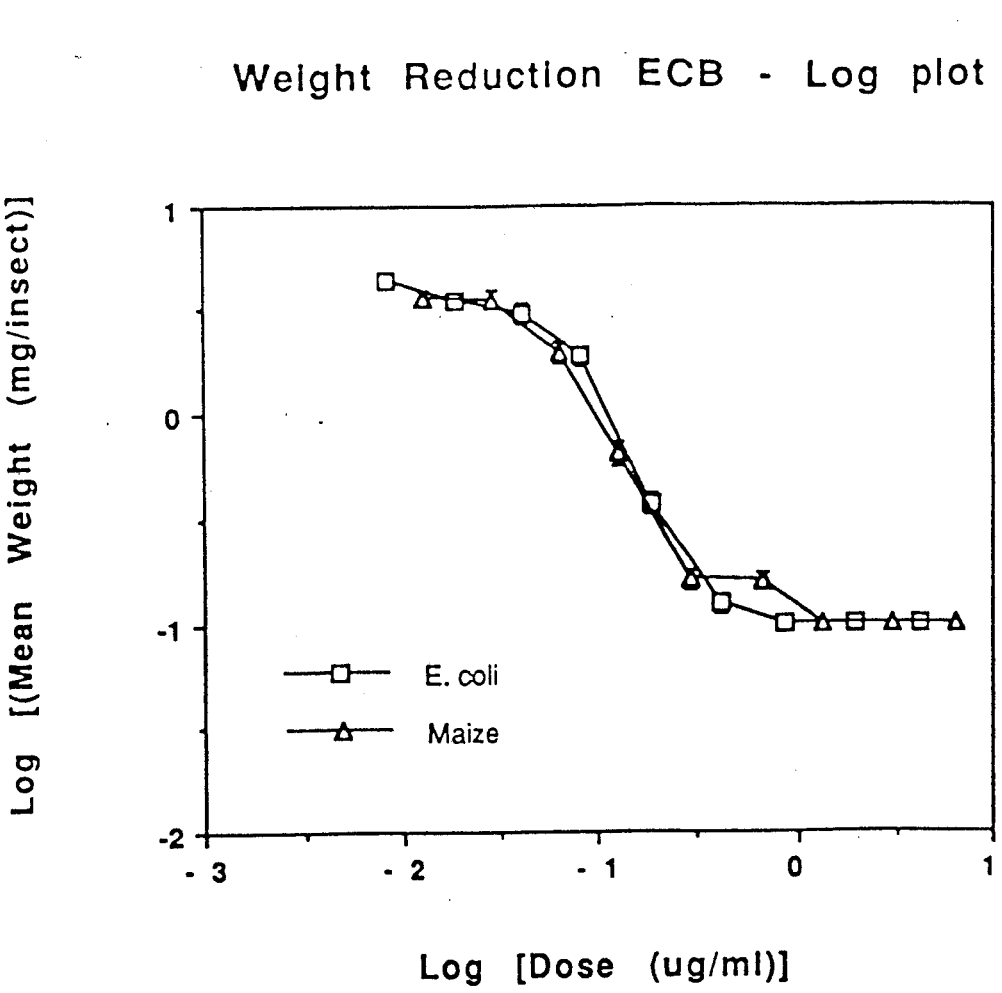


Figure 7b: European Corn Borer Sensitivity to HD-1tMz and HD-1t - Weight. Data from figure 7a expressed as log of mean weight vs log of dose. Bars represent standard errors.

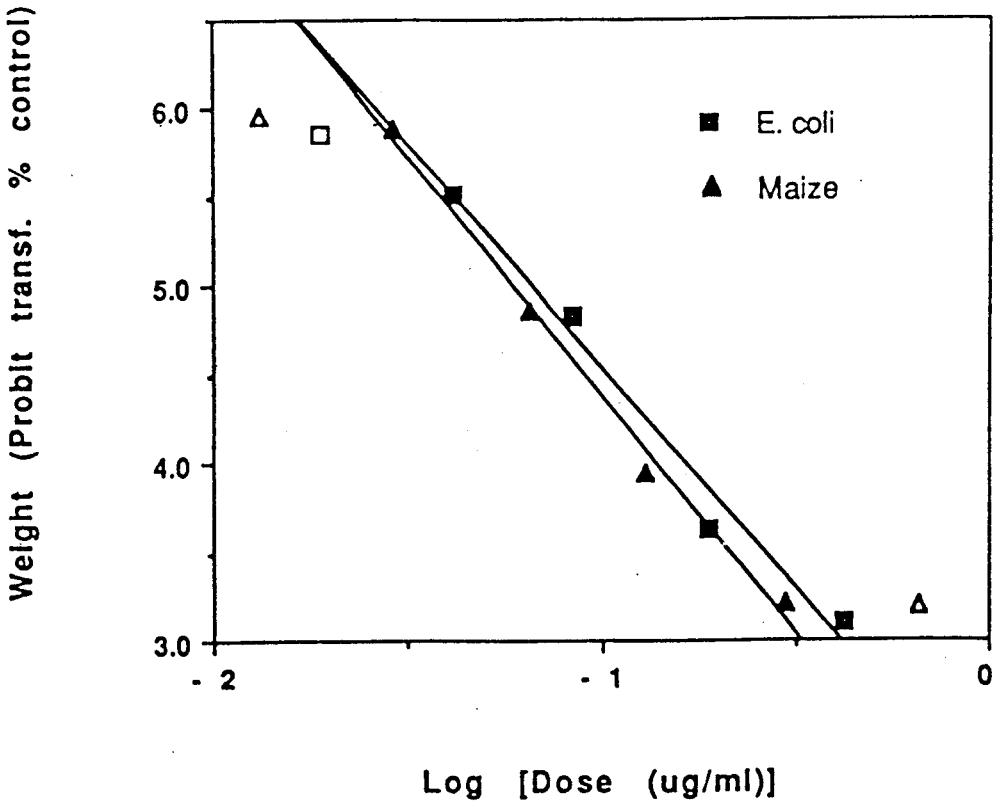


Figure 7c: European Corn Borer Sensitivity to HD-1tMz and HD-1t - Weight. Data from figure 7a expressed as % control weight and transformed using Probit tables. Lines represent regression fit to the solid data points. EC_{50} values presented in the text were calculated from these lines.

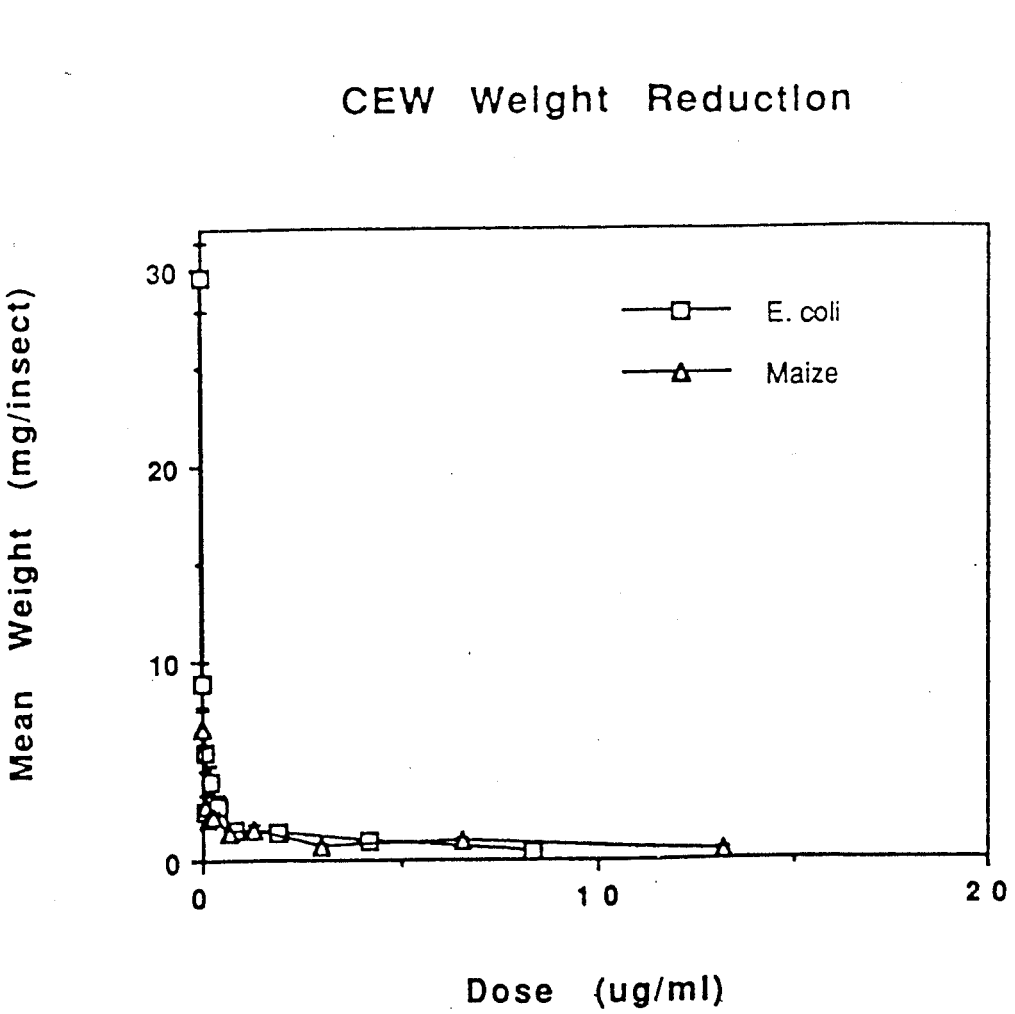


Figure 8a: Corn Earworm Sensitivity to HD-1tMz and HD-1t - Weight. Weights were taken of survivors after 5 days. Each data point represents mean weight of the survivors from the initial 30 insects treated at each dose. Bars represent standard errors.

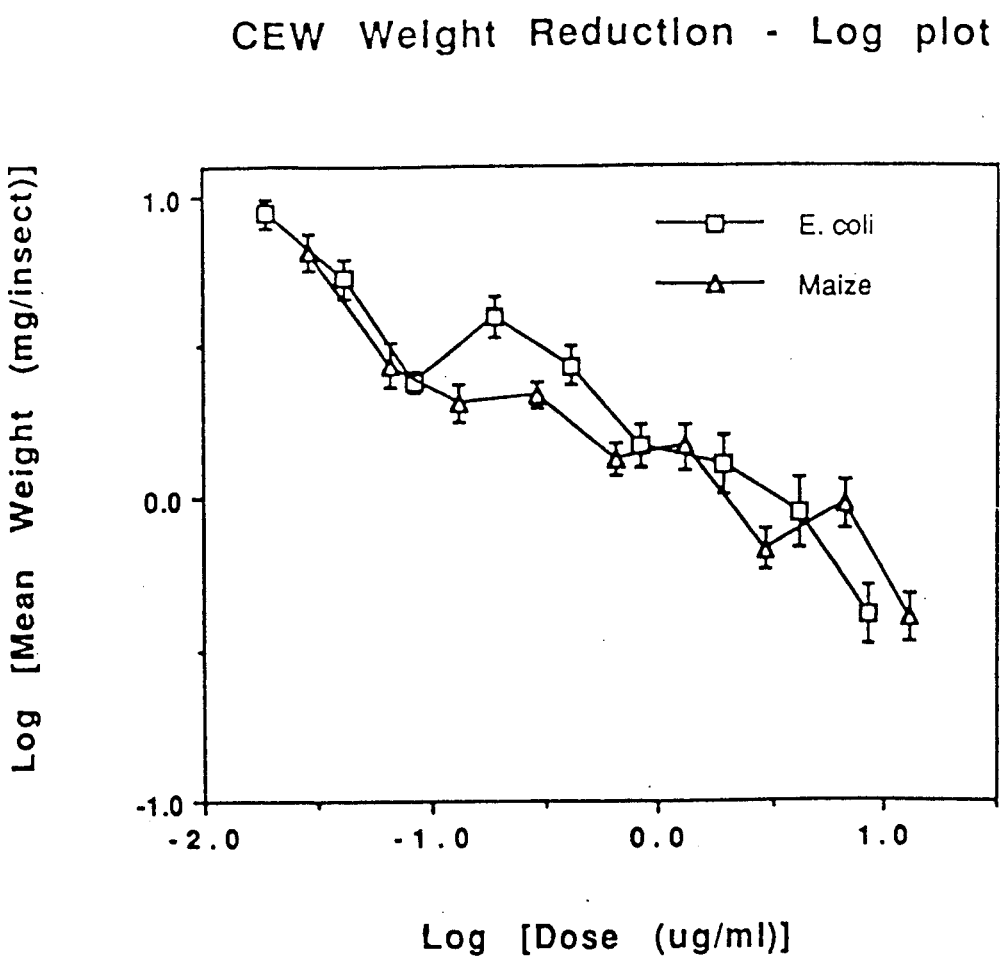


Figure 8b: Corn Earworm Sensitivity to HD-1tMz and HD-1t - Weight. Data from figure 8a expressed as log of mean weight vs log of dose. Bars represent standard errors.

Attachment 1

==PC/GENE===

```
*******************************************
* TRANSLATION OF A NUCLEIC ACID SEQUENCE *
*******************************************

Done on DNA sequence pZO1502
Total number of bases is: 1845.
Analysis done on the complete sequence.
Done on (absolute) phase(s): 1.
Using the Universal genetic code.

                    ------------------------------------

          10         20         30         40         50         60
           |          |          |          |          |          |
ATGGACAACAACCCAAACATCAACGAATGCATTCCATACAACTGCTTGAGTAACCCAGAA
 M  D  N  N  P  N  I  N  E  C  I  P  Y  N  C  L  S  N  P  E

          70         80         90        100        110        120
           |          |          |          |          |          |
GTTGAAGTACTTGGTGGAGAACGCATTGAAACCGGTTACACTCCCATCGACATCTCCTTG
 V  E  V  L  G  G  E  R  I  E  T  G  Y  T  P  I  D  I  S  L

         130        140        150        160        170        180
           |          |          |          |          |          |
TCCTTGACACAGTTTCTGCTCAGCGAGTTCGTGCCAGGTGCTGGGTTCGTTCTCGGACTA
 S  L  T  Q  F  L  L  S  E  F  V  P  G  A  G  F  V  L  G  L

         190        200        210        220        230        240
           |          |          |          |          |          |
GTTGACATCATCTGGGGTATCTTTGGTCCATCTCAATGGGATGCATTCCTGGTGCAAATT
 V  D  I  I  W  G  I  F  G  P  S  Q  W  D  A  F  L  V  Q  I

         250        260        270        280        290        300
           |          |          |          |          |          |
GAGCAGTTGATCAACCAGAGGATCGAAGAGTTCGCCAGGAACCAGGCCATCTCTAGGTTG
 E  Q  L  I  N  Q  R  I  E  E  F  A  R  N  Q  A  I  S  R  L

         310        320        330        340        350        360
           |          |          |          |          |          |
GAAGGATTGAGCAATCTCTACCAAATCTATGCAGAGAGCTTCAGAGAGTGGGAAGCCGAT
 E  G  L  S  N  L  Y  Q  I  Y  A  E  S  F  R  E  W  E  A  D

         370        380        390        400        410        420
           |          |          |          |          |          |
CCTACTAACCCAGCTCTCCGCGAGGAAATGCGTATTCAATTCAACGACATGAACAGCGCC
 P  T  N  P  A  L  R  E  E  M  R  I  Q  F  N  D  M  N  S  A
```

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        430         440         450         460         470         480
         |           |           |           |           |           |
TTGACCACAGCTATCCCATTGTTCGCAGTCCAGAACTACCAAGTTCCTCTCTTGTCCGTG
 L  T  T  A  I  P  L  F  A  V  Q  N  Y  Q  V  P  L  L  S  V

        490         500         510         520         530         540
         |           |           |           |           |           |
TACGTTCAAGCAGCTAATCTTCACCTCAGCGTGCTTCGAGACGTTAGCGTGTTTGGGCAA
 Y  V  Q  A  A  N  L  H  L  S  V  L  R  D  V  S  V  F  G  Q

        550         560         570         580         590         600
         |           |           |           |           |           |
AGGTGGGGATTCGATGCTGCAACCATCAATAGCCGTTACAACGACCTTACTAGGCTGATT
 R  W  G  F  D  A  A  T  I  N  S  R  Y  N  D  L  T  R  L  I

        610         620         630         640         650         660
         |           |           |           |           |           |
GGAAACTACACCGACCACGCTGTTCGTTGGTACAACACTGGCTTGGAGCGTGTCTGGGGT
 G  N  Y  T  D  H  A  V  R  W  Y  N  T  G  L  E  R  V  W  G

        670         680         690         700         710         720
         |           |           |           |           |           |
CCTGATTCTAGAGATTGGATTAGATACAACCAGTTCAGGAGAGAATTGACCCTCACAGTT
 P  D  S  R  D  W  I  R  Y  N  Q  F  R  R  E  L  T  L  T  V

        730         740         750         760         770         780
         |           |           |           |           |           |
TTGGACATTGTGTCTCTCTTCCCGAACTATGACTCCAGAACCTACCCTATCCGTACAGTG
 L  D  I  V  S  L  F  P  N  Y  D  S  R  T  Y  P  I  R  T  V

        790         800         810         820         830         840
         |           |           |           |           |           |
TCCCAACTTACCAGAGAAATCTATACTAACCCAGTTCTTGAGAACTTCGACGGTAGCTTC
 S  Q  L  T  R  E  I  Y  T  N  P  V  L  E  N  F  D  G  S  F

        850         860         870         880         890         900
         |           |           |           |           |           |
CGTGGTTCTGCCCAAGGTATCGAAGGCTCCATCAGGAGCCCACACTTGATGGACATCTTG
 R  G  S  A  Q  G  I  E  G  S  I  R  S  P  H  L  M  D  I  L

        910         920         930         940         950         960
         |           |           |           |           |           |
AACAGCATAACTATCTACACCGATGCTCACAGAGGAGAGTATTACTGGTCTGGACACCAG
 N  S  I  T  I  Y  T  D  A  H  R  G  E  Y  Y  W  S  G  H  Q

        970         980         990        1000        1010        1020
         |           |           |           |           |           |
ATCATGGCCTCTCCAGTTGGATTCAGCGGGCCCGAGTTTACCTTTCCTCTCTATGGAACT
 I  M  A  S  P  V  G  F  S  G  P  E  F  T  F  P  L  Y  G  T
```

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      1030        1040        1050        1060        1070        1080
        |           |           |           |           |           |
ATGGGAAACGCCGCTCCACAACAACGTATCGTTGCTCAACTAGGTCAGGGTGTCTACAGA
 M  G  N  A  A  P  Q  Q  R  I  V  A  Q  L  G  Q  G  V  Y  R

      1090        1100        1110        1120        1130        1140
        |           |           |           |           |           |
ACCTTGTCTTCCACCTTGTACAGAAGACCCTTCAATATCGGTATCAACAACCAGCAACTT
 T  L  S  S  T  L  Y  R  R  P  F  N  I  G  I  N  N  Q  Q  L

      1150        1160        1170        1180        1190        1200
        |           |           |           |           |           |
TCCGTTCTTGACGGAACAGAGTTCGCCTATGGAACCTCTTCTAACTTGCCATCCGCTGTT
 S  V  L  D  G  T  E  F  A  Y  G  T  S  S  N  L  P  S  A  V

      1210        1220        1230        1240        1250        1260
        |           |           |           |           |           |
TACAGAAAGAGCGGAACCGTTGATTCCTTGGACGAAATCCCACCACAGAACAACAATGTG
 Y  R  K  S  G  T  V  D  S  L  D  E  I  P  P  Q  N  N  N  V

      1270        1280        1290        1300        1310        1320
        |           |           |           |           |           |
CCACCCAGGCAAGGATTCTCCCACAGGTTGAGCCACGTGTCCATGTTCCGTTCCGGATTC
 P  P  R  Q  G  F  S  H  R  L  S  H  V  S  M  F  R  S  G  F

      1330        1340        1350        1360        1370        1380
        |           |           |           |           |           |
AGCAACAGTTCCGTGAGCATCATCAGAGCTCCTATGTTCTCATGGATTCATCGTAGTGCT
 S  N  S  S  V  S  I  I  R  A  P  M  F  S  W  I  H  R  S  A

      1390        1400        1410        1420        1430        1440
        |           |           |           |           |           |
GAGTTCAACAATATCATTCCTTCCTCTCAAATCACCCAAATCCCATTGACCAAGTCTACT
 E  F  N  N  I  I  P  S  S  Q  I  T  Q  I  P  L  T  K  S  T

      1450        1460        1470        1480        1490        1500
        |           |           |           |           |           |
AACCTTGGATCTGGAACTTCTGTCGTGAAAGGACCAGGCTTCACAGGAGGTGATATTCTT
 N  L  G  S  G  T  S  V  V  K  G  P  G  F  T  G  G  D  I  L

      1510        1520        1530        1540        1550        1560
        |           |           |           |           |           |
AGAAGAACTTCTCCTGGCCAGATTAGCACCCTCAGAGTTAACATCACTGCACCACTTTCT
 R  R  T  S  P  G  Q  I  S  T  L  R  V  N  I  T  A  P  L  S

      1570        1580        1590        1600        1610        1620
        |           |           |           |           |           |
CAAAGATATCGTGTCAGGATTCGTTACGCATCTACCACAAACTTGCAATTCCACACCTCC
 Q  R  Y  R  V  R  I  R  Y  A  S  T  T  N  L  Q  F  H  T  S
```

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      1630       1640       1650       1660       1670       1680
        |          |          |          |          |          |
ATCGACGGAAGGCCTATCAATCAGGGTAACTTCTCCGCAACCATGTCAAGCGGCAGCAAC
 I  D  G  R  P  I  N  Q  G  N  F  S  A  T  M  S  S  G  S  N

      1690       1700       1710       1720       1730       1740
        |          |          |          |          |          |
TTGCAATCCGGCAGCTTCAGAACCGTCGGTTTCACTACTCCTTTCAACTTCTCTAACGGA
 L  Q  S  G  S  F  R  T  V  G  F  T  T  P  F  N  F  S  N  G

      1750       1760       1770       1780       1790       1800
        |          |          |          |          |          |
TCAAGCGTTTTCACCCTTAGCGCTCATGTGTTCAATTCTGGCAATGAAGTGTACATTGAC
 S  S  V  F  T  L  S  A  H  V  F  N  S  G  N  E  V  Y  I  D

      1810       1820       1830       1840
        |          |          |          |
CGTATTGAGTTTGTGCCTGCCGAAGTTACCTTCGAGGCTGAGTAC
 R  I  E  F  V  P  A  E  V  T  F  E  A  E  Y
```

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