96-291-01p

DEKALB Genetics Corporation
DISCOVERY RESEARCH, 62 MARITIME DRIVE, MYSTIC, CT 06355-1958
203/572-5200 FAX: 203/572-5240

October 16, 1996

Dr. Subash C. Gupta
Biotechnologist
Biotechnology Permits
BBEP
USDA-APHIS
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Subject: **Petition for Determination of Nonregulated Status: Insect Protected Corn *(Zea mays* L.) with the *cryIA(c)* Gene from *Bacillus thuringiensis* subsp. *kurstaki***

Dear Dr. Gupta:

Enclosed is a resubmission of a Petition for Determination of Nonregulated Status for Insect Protected Corn *(Zea mays* L.) with the *cryIA(c)* Gene from *Bacillus thuringiensis* subsp. *kurstaki.* Two original copies of the petition are enclosed. The petition requests that insect protected corn line DBT418 no longer be considered a regulated article under regulations in 7 CFR part 340.

Transgenic corn line DBT418 has been field tested since 1993 under USDA-APHIS notifications and in 1996 under an Experimental Use Permit (69575-EUP-1) granted by the EPA. Results from these field tests have demonstrated that insect protected corn line DBT418 is protected season long from leaf and stalk feeding damage caused by European corn borer (*Ostrina nubalis*).

In addition to the information that we provided at the request of APHIS in the form of replacement pages to the original petition, in the resubmission we have included additional information on the effect of insect protected corn line DBT418 on nontarget lepidopteran insect species such as corn earworm, Southwestern corn borer, and fall armyworm. We have provided statistical analysis of the data where possible and paid careful attention to consistency in the description of the product and its effect on nontarget lepidopteran insects. We have also included a detailed description of the

phenotype of DBT418 as relates to control of European corn borer, demonstrating the utility and benefit of the product.

As we have discussed, APHIS-USDA approval in early 1997 (February) is crucial to a successful product launch in 1997 for DEKALB. We will continue to do everything possible to provide the information necessary for deregulation of insect protected corn line DBT418. Thank you for your cooperation.

Sincerely,

T. Michael Spencer
Manager, Regulatory Affairs

cc: C. Flick, DEKALB
M. Stephens, DEKALB
C. Mackey, DEKALB

96-291-01p

Petition for Determination of Nonregulated Status:

Insect Protected Corn *(Zea mays* L.) with the *cryIA(c)* Gene from *Bacillus thuringiensis* subsp. *kurstaki*

DEKALB #: DGC 96-059P

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

Submitted by:

T. Michael Spencer, Regulatory Affairs Manager
DEKALB Genetics Corporation
62 Maritime Drive
Mystic, CT 06355
Tel: (860) 572-5200
Fax: (860) 572-5240

On Behalf of:

DEKALB Genetics Corporation
3100 Sycamore Road
DeKalb, IL 60115

October 14, 1996

Prepared by:

T. Michael Spencer, Emil M. Orozco, Rebecca M. Doyle

Contributors:

W.R. Adams, J.B. Beavers, M.K. Collins, N.A. Garvey, D.E. Kruger, L. Lacetti, N. LaPietra, T.N. Merriman, R.D. Millham, J. Nutkis, E.M. Orozco, S.J. Palmer, M.A. Stephens, C.S. Vetsch, D.A. Walters, D.S. Walters, L.C. Wilson

Petition For Determination of Nonregulated Status for Insect Protected Corn *(Zea mays* L.) with the *cryIA(c)* gene from *Bacillus thuringiensis* subsp. *kurstaki.*

Summary

DEKALB Genetics Corporation is submitting this Petition for Determination of Nonregulated Status to the Animal Plant Health Inspection Service (APHIS) regarding corn which contains and expresses a *cryIA(c)* gene derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.*). This corn line also contains and expresses the herbicide resistance gene *bar* derived from *Streptomyces hygroscopicus*. This petition requests a determination from APHIS that insect protected corn line DBT418, any progenies derived from crosses between DBT418 and traditional corn varieties, and any progeny derived from crosses of DBT418 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under regulations in 7 CFR part 340.

Corn is the largest crop in the United States in terms of planted acreage, total production, and crop value. Corn production in 1993 was 161 million metric tons produced on over sixty million acres in the U.S. The majority of national production is concentrated across what is known as the "Corn Belt" in the upper Midwest. The European corn borer (ECB), *Ostrinia nubilalis*, causes severe economic damage as it feeds on leaf and stalk tissue compromising the structural integrity of the corn plant. This feeding damage leads to plant lodging and yield loss. Currently available chemical insecticides offer limited protection from ECB damage because applications must be made prior to the time the insect bores into the stalk and repeated applications are often necessary. ECB is one of the most important pests of corn in the United States, with estimates of yield loss due to ECB damage averaging five to ten percent of corn produced annually.

DEKALB has developed genetically modified corn plants that effectively control ECB. These genetically modified corn plants produce an insect control protein, CryIA(c), derived from the common soil bacterium *Bacillus thuringiensis* subsp. *kurstaki (B.t.)*. Microbial formulations containing insecticidal *B.t.* proteins have been registered by the EPA and commercially available for over thirty years. The protein produced by insect protected corn is identical to the insecticidally active, trypsin resistant core of CryIA(c) protein that is found in nature and in commercial *B.t.* formulations registered as pesticides with the EPA. This protein is highly selective in controlling lepdopteran insects and is expressed at an effective level in plant tissue throughout the growing season.

Transgenic corn line DBT418 also contains and expresses the *bar* gene, originally isolated from the soil bacterium *Streptomyces hygroscopicus*. The *bar* gene encodes phosphinothricin acetyltransferase (PAT), an enzyme that confers resistance to the herbicide glufosinate. In 1995, DEKALB petitioned USDA/APHIS for Determination of Nonregulated Status for a transgenic

corn line designated B16 which also contains and expresses the *bar* gene (petition no. 95-145-01). B16 was created using a plasmid designated pDPG165, the same *bar*-containing plasmid used to create the insect-protected line that is the subject of this petition. B16 was granted nonregulated status by USDA/APHIS on December 19, 1995. DEKALB also completed a consultation with the FDA concerning B16 (also known as DLL25) on March 8, 1996 in which the FDA stated that it had "...no further questions concerning grain, fodder, or silage from event DLL25 corn...".

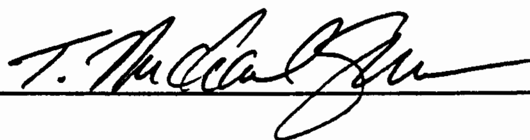
The insect protected corn line, DBT418, has been field tested in the U.S. by DEKALB since 1993 under APHIS notifications. In addition, the DBT418 line has been field tested in Argentina, France, Italy and Canada. An Experimental Use Permit (69575-EUP-1) was obtained from the U.S. EPA in 1996 to perform larger scale testing of insect protected corn. Results from field experiments to date have demonstrated that corn line DBT418 is protected from ECB damage throughout the season. Growers planting insect protected corn will not require insecticide applications to control ECB. This reduction in insecticide use will enhance biological control and the implementation of other pest management strategies for other corn pests. In addition, these plants exhibit no pathogenic properties, are no more likely to become weeds than the non-modified parental corn lines, are unlikely to increase the weediness potential for any other cultivated plants or native species, and are equivalent morphologically, agronomically, and compositionally to the parental corn lines. An application to register DBT418 as a "plant pesticide", as well as applicable tolerance petitions, were filed with the EPA earlier this year.

Insect protected corn offers several ecological advantages as compared to conventional insecticides. The CryIA(c) protein is ecologically benign, i.e., it breaks down rapidly in the soil, and is safe to non-target organisms such as fish, birds, mammals, and beneficial insects. Insect protected corn offers decreased pesticide exposure risk for manufacturing and farm workers, and is an ideal fit with Integrated Pest Management (IPM) and sustainable agricultural systems. In addition, the risk of an uncontrolled introduction of this corn into the environment through hybridization or outcrossing to native species is virtually non-existent in the U.S.

In conclusion, for reasons stated above, DEKALB Genetics Corporation requests a determination from APHIS that insect protected corn line DBT418 and any progenies derived from crosses between DBT418 and other corn varieties no longer be considered regulated articles under regulations in 7 CFR part 340.

Certification

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



T. Michael Spencer, Regulatory Affairs Manager
DEKALB Genetics Corporation
62 Maritime Drive
Mystic, CT 06355
Tel: (860) 572-5200
Fax: (860) 572-5240

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I. Rationale for Development of *B.t.* Corn

A. Need for Insect Protected Corn

Corn is the largest U.S. crop in terms of acreage, total production, and crop value (National Corn Growers Association, 1994). European corn borer (ECB), *Ostrinia nubilalis* (Hübner), is among the most important corn insect pests in the U.S. and worldwide (Dickie and Guthrie, 1988). This pest ranges from the Eastern seashore west to the Rocky Mountains and from southern Canada to Florida and the Gulf States. In the central corn belt, the pest typically completes two generations each year, but in warm years may complete a partial to full third generation (USDA, 1992). ECB damage occurs as a result of leaf feeding, stalk tunneling, leaf sheath and collar feeding, and ear damage (USDA, 1992). Researchers from across the pest's geographic range have estimated a five to ten percent corn yield loss annually, attributable to ECB damage (Bode and Calvin, 1990; Guthrie et al., 1975; Rice, 1994a-c). Yield losses are attributed to disruption of nutrient and water translocation to key tissues, secondary disease infections, stalk lodging, ear droppage and kernel damage.

Control of ECB using conventional insecticide applications is variable due to difficulties in the proper timing of the application and placement of the insecticide where ECB larvae are feeding. Deviations from the optimal date for applying an insecticide can result in significantly less control and more than one insecticide application may be necessary. Currently, a field scouting program is required to time insecticide applications properly (USDA, 1992). Conventional hybrids offering resistance to the first generation (leaf-feeding resistance) ECB are also used to minimize the yield loss due to ECB damage. However, to date, these hybrids do not have the yield potential of susceptible full-season hybrids (USDA, 1992). It has been estimated that corn growers using insect protected corn instead of conventional insecticides for ECB control could experience savings of $50 million annually. Savings on insecticides, coupled with increased yields, could lead to an annual benefit of between 1 and 1.5 billion dollars (Croon et al., 1995).

DEKALB has developed genetically modified corn plants that provide resistance to ECB. This insect protected corn offers a new mechanism to produce and deliver a highly effective insecticide to target pests (e.g. production by cells of the crop plant, rather than industrial production and application by spray equipment). Resistance to ECB is conferred by the *cryIA(c)* gene isolated from *Bacillus thuringiensis* subsp. *kurstaki*. The CryIA(c) protein that is produced in DEKALB's insect protected corn is very specific in its activity, exhibiting toxicity only to lepidopteran insects.

B. Benefits of Insect Protected Corn and Impact on Agricultural Practices

Insect protected corn provides excellent control of an insect, ECB, that causes significant decreases in corn yields every year in North America. Results from field experiments with

DEKALB's genetically modified corn expressing CryIA(c) protein, performed under APHIS notification in 1993, 1994, and 1995, revealed a high level of efficacy against ECB. Thus, growers planting insect protected corn will not require insecticide applications to control ECB. This reduction in insecticide use will enhance biological control and the implementation of other pest management strategies for other corn pests. Also, the insect protected corn plants exhibit no pathogenic properties, are no more likely to become weeds than the non-modified parental corn lines, are unlikely to increase the weediness potential for any other cultivated plants or native species, and are equivalent morphologically, agronomically, and compositionally to the parental corn lines.

The use of insect protected corn will have a positive impact on the environment and on current agricultural practices. As described above, control of ECB using conventional insecticide applications, including organophosphates, synthetic pyrethroids, and microbial *B.t.*, is inconsistent due to difficulties in the proper timing of the application and directing the insecticide to tissues on which ECB larvae are feeding. Insect protected corn provides a more economical and less labor intensive means to control ECB. Insect protected corn will also lead to reduction in the manufacturing, shipment, storage, and use of chemical insecticides. Insect protected corn will also lead to a reduction of exposure of workers to potentially hazardous pesticides. Insect protected corn does not require special equipment or machinery, and is therefore, equally beneficial to large and small growers.

As mentioned above, CryIA(c) protein is very specific in its activity against lepidopteran insects, and is safe to non-target organisms such as fish, birds, mammals, and beneficial non-lepidopteran insects (EPA, 1988; see section VIII.A). Insect protected corn line DBT418 has been shown to offer good control of ECB as well as significant control of Southwestern corn borer (*Diatraea grandiosella*). Growth inhibition of Corn earworm (*Heliothis zea*) has been demonstrated as the result of DBT418 silk and ear feeding. Little or no inhibition of Fall armyworm (*Spodoptera frugiperda*) feeding has been observed on DBT418 plants. A summary of the effect of DBT418 on European corn borer, Southwestern corn borer, Corn earworm, and Fall armyworm is presented in Appendix IV. DEKALB is working with the EPA to develop an insect resistance management plan (see section VIII.F) focused on deploying an insect protected corn product in a manner consistent with maintaining the utility of *B.t.k.* proteins for insect protection in corn and other crops. Insect resistance management strategies for primary and secondary lepidopteran pests are being developed.

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. Insect protected corn fits well with an Integrated Pest Management (IPM) systems given lack of toxicity to many beneficial insects. Insect protected corn offers selective activity against important pests of corn without disrupting pest suppression by natural enemies, such as parasites and predators. Insect protected corn will have significant benefits to the grower, the general public, and the environment.

C. Needs for, and Benefits of Herbicide Resistant Corn

As mentioned above in the summary, transgenic corn line DBT418 also contains and expresses the *bar* gene, originally isolated from the soil bacterium *Streptomyces hygroscopicus*. The *bar* gene encodes phosphinothricin acetyltransferase, which confers resistance to glufosinate, the active ingredient in Finale®, Rely®, Basta®, and Liberty® herbicides. In 1995 DEKALB petitioned USDA/APHIS for determination of nonregulated status of transgenic corn line B16 (also known as DLL25) which contains and expresses *bar* and is resistant to glufosinate herbicides. In this document (petition no. 95-145-01), the needs for and benefits of glufosinate resistant corn were presented and discussed in detail. The *bar* plasmid, pDPG165, used to create transgenic corn line B16, is the same plasmid used to create insect protected corn line DBT418.

D. Regulatory Approvals

Before commercializing the insect and herbicide resistant corn line DBT418 in the U.S., DEKALB will seek the following regulatory approvals:

1. This determination from USDA/APHIS that insect protected corn line DBT418, and all progenies from crosses between insect protected corn line DBT418 and other corn varieties, is no longer a regulated article according to 7 CFR 340.6.

2. Regulatory approval from the Environmental Protection Agency (EPA) of the CryIA(c) insecticidal protein as expressed in insect protected corn under the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). This application has been submitted.

3. An exemption from the requirement of a tolerance for the CryIA(c) insecticidal protein and phosphinothricin acetyltransferase (PAT) protein, and the genetic materials necessary for the production of these proteins in or on all agricultural commodities under sections 408 of the Federal Food Drug and Cosmetic Act (FFDCA) from the EPA.

In addition, prior to commercialization, we will complete consultations with the FDA under their May 29, 1992 policy statement concerning foods derived from new plant varieties.

DEKALB will consult with the pesticide and, if applicable, biotechnology regulatory officials of the states in which the commercial product will be sold and obtain a state license if such is required.

II. The Corn Family

A. History of the Crop

Corn is a native American species. The species *Zea mays* was probably produced as a result of domestication of the wild species teosinte, *Zea Mexicana* about 8,000 years ago in Mexico. Since that time corn has been continuously cultivated in the Americas and following the arrival of Europeans in the Americas in the sixteenth century spread throughout the world. Three deliberate steps occurred in the domestication of corn from teosinte according to Galinat (1983): (1) the development of paired female spikelets in corn in comparison to solitary spikelets in teosinte; (2) a many ranked central spikelet in corn in comparison to a two ranked central spikelet in teosinte; and (3) a non-shattering rachis (cob) in corn in comparison to a shattering cob in teosinte. Each of these advances made corn less adaptive to survival in the wild and the total combination makes survival in the wild highly unlikely.

The first commercial hybrids of corn were produced in the 1920s and were rapidly accepted during the 1930s and 1940s. Development of hybrids adapted to almost every corn growing area of the United States has led to the growing of corn outside the Corn Belt. From 1975-1991, planted corn acreage in the United States ranged from about 60 to 85 million acres. Harvested crops yielded about 4.2 to 8.9 billion bushels of grain. The value of the 1991 crop was about $18 billion on about 7.5 billion bushels of grain.

B. Taxonomy of the Genus *Zea*

Corn is a grass of the family *Gramineae* belonging to the tribe *Maydeae* which includes seven genera. Five genera are native to India to Burma through the East Indies to Australia: *Coix, Schlerachne, Polytoca, Chinonachne,* and *Trilohachne*. Two genera, however, are native to the Americas: *Zea* and *Tripsacum* (gamagrass). Teosinte species including *Zea Mexicana* (an annual species) and *Zea perennis* and *Zea diploperennis* (perennial species) are found in Mexico and Guatemala and will hybridize with cultivated *Zea mays*. *Tripsacum*, includes about 16 species, is found from Mexico to Brazil and in the United States. *T. dactyloides* is found in the Eastern and Central United States. *T. lanceolatum* occurs in the Southwest and *T. floridanum* is native to South Florida. *Tripsacum* can be hybridized to *Zea mays* under experimental conditions, but the progeny are generally sterile or have greatly reduced fertility. It is unlikely that *Tripsacum* played any role in the development of corn. *Zea mays* is believed to have developed as a species as a result of domestication and is the only cultivated crop in the *Maydeae* tribe.

C. Genetics of Corn

Corn is the genetically most characterized higher plant species. *Zea mays* has 10 pairs of chromosomes. An extensive genetic map of *Zea mays* has been constructed containing morphological, enzyme and DNA markers (e.g. Maize Genetics Cooperation Newsletter 68, 1994). Diploid and tetraploid teosintes are known, e.g. *Z. perennis* (2n = 40) and *Z. diploperennis* (2n = 20). Hundreds of morphological and DNA markers have been mapped in maize and hundreds of mutants are known (e.g., see Maize Genetics Cooperation Newsletter, 1994, for recent genetic map of corn). Teosinte and corn have extensive cytogenetic homology. F_1 hybrids are fertile and chromosomes pair closely at pachytene and corn-teosinte hybrids have the same frequencies of crossing over as in corn itself. Although teosinte and corn are not cross-sterile, the species are reproductively isolated by flowering time (teosinte flowers later), geographic distribution, and block inheritance (linked groups of genes controlling the teosinte-like or corn-like female inflorescence co-segregate and when linkages are broken combinations are generated that are neither adapted to cultivation or the wild). No one of these factors leads to complete reproductive isolation, but the combination of three makes it unlikely that cross-hybridization will occur.

D. Reproductive Biology

Early in vegetative plant growth the tassel begins to develop and when it attains full size bears staminate flowers which produce pollen. The tassel dies following pollen shed. At the same time that tassel development begins branches of the stem develop, most of which develop into ear shoots. Most of the lower ear shoots degenerate, but those that develop produce functional female flowers. The ovary is attached to a long silk which catches the pollen as it is shed. The pollen tube penetrates the silk and grows to the embryo sac where a double fertilization occurs. The fertilized egg develops into the zygote. The second fertilization is the fertilization of one of the two polar nuclei in the embryo sac. Following fertilization of one polar nucleus, it fuses with the other polar nucleus to become the triploid endosperm nucleus. The endosperm comprises the bulk of the mature kernel. During germination the embryo develops into a seedling and consumes the endosperm.

III. Description of the Transformation System and the Plasmids Utilized

A. Transformation of Corn via Particle Acceleration

Plasmid DNA was introduced into embryogenic corn cells using microprojectile bombardment (Gordon-Kamm et al., 1990). The cell culture was initiated from immature embryos of an inbred line designated AT824. Transformants were selected by their ability to survive and grow in the presence of the herbicide bialaphos (bialaphos, which contains phosphinothricin, is a tripeptide fermentation product of *Streptomyces hygroscopicus*). A transgenic callus line, designated DBT418, was selected on medium containing bialaphos. Plants were regenerated from embryogenic DBT418 callus by placing the callus on media which stimulate the production of shoots and roots. Regenerated DBT418 plants (R_0 generation) were crossed with control, non-transgenic, inbred corn lines to produce R_1 seed that inherited the inserted transgenes. Repeated backcrossing to various inbred lines was performed to create hybrid germplasm containing the DBT418 insertion event. During the backcross program, segregating populations of plants were sprayed with glufosinate herbicide to identify positive segregants. Typically, about one-half of the plants resulting from a backcross were resistant to the herbicide. The herbicide resistant plants were also resistant to infestations of ECB, indicating that the herbicide resistance and insect resistance genes were linked, probably resulting from the same insertion event.

B. Description of the Plasmids Used for Transformation

1. pDPG699

The basic constituents of the *cryIA(c)* expression vector are shown in Figure III.1 and Table III.1. The *cryIA(c)* gene in pDPG699 is under control of a chimeric OCS-35S promoter. The OCS element is a 20 base pair consensus sequence of DNA that is found in a variety of strong promoters (Bouchez et al., 1989). The double copy of the OCS sequence was positioned upstream of a 90 base pair segment of the Cauliflower Mosaic Virus 35S promoter (Odell et al., 1985). The *cryIA(c)* gene in pDPG699 is terminated by the 3'-end from the potato protease inhibitor gene (Thornburg et al., 1987).

2. pDPG165

Also introduced into transgenic corn line DBT418 was a plasmid, pDPG165, which contains the *bar* gene. The *bar* gene is comprised of about 549 base pairs encoding the PAT protein of 183 amino acids with a molecular weight of about 22,000. The gene has been completely sequenced (White et al., 1990). A map of pDPG165 is shown in Figure III.1 and the constituents of the plasmid are summarized in Table III.2. The *bar* gene in pDPG165 is under control of an approximately 830 base pair fragment of the Cauliflower Mosaic Virus promoter and the transcript 7 (.) 3'-end from *Agrobacterium tumefaciens* (Dhaese et al., 1983).

3. pDPG320

In DNA preparations used to produce DBT418, pDPG320, a vector containing the protease inhibitor II gene from potato (*pinII*) was also included. A map of pDPG320 is shown in Figure III.1, and the constituents of the plasmid are summarized in Table III.3. The potato *pinII* gene in pDPG320 is under control of a 420 fragment of the Cauliflower Mosaic Virus promoter in combination with *adh1* intron 1 from maize (Dennis et al., 1984). The potato *pinII* gene in pDPG320 is terminated by the potato *pinII* 3'-end.

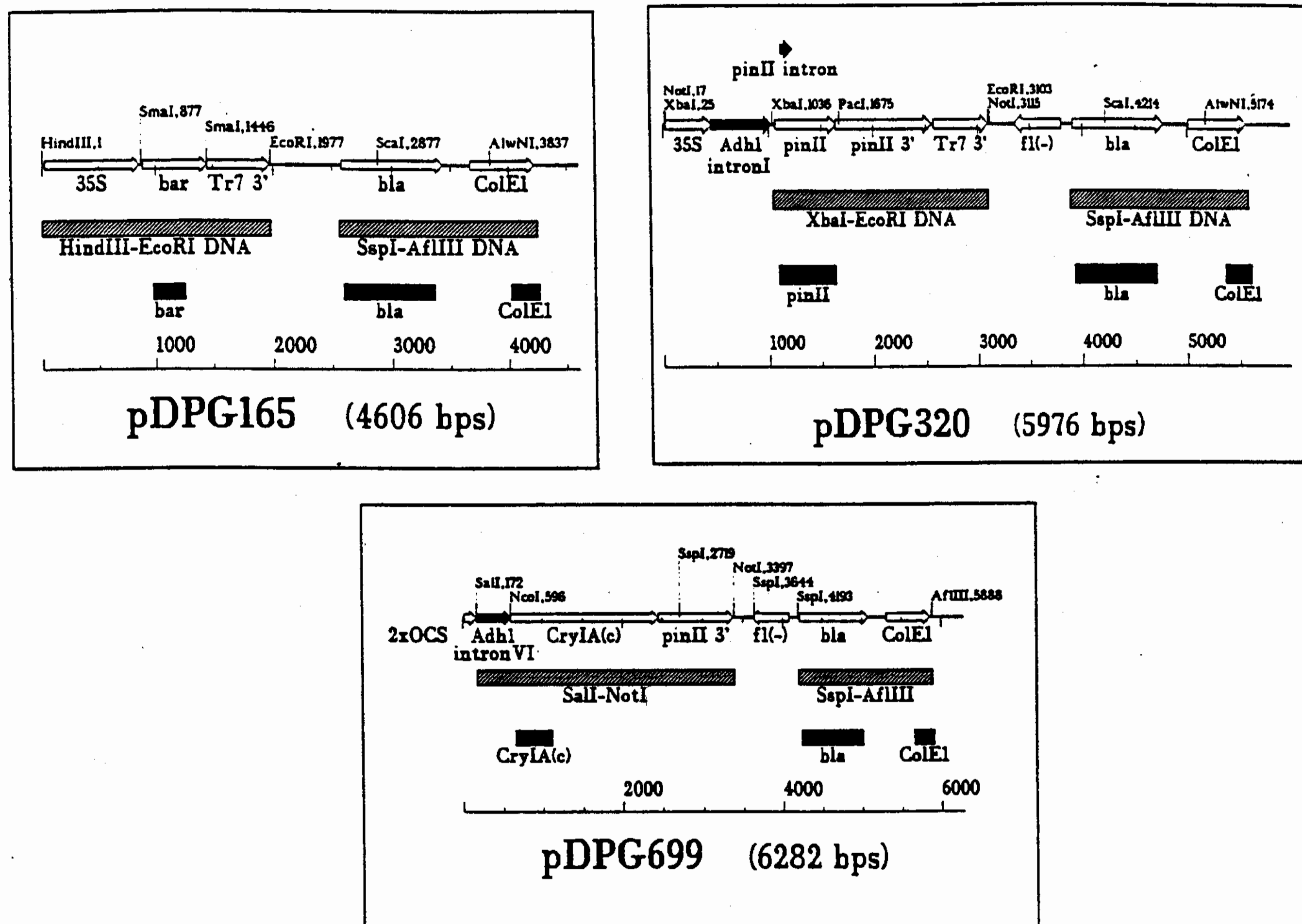


Figure III.1. Plasmid Maps of pDPG165, pDPG320, and pDPG699. Each plasmid map is shown in linear format with arrows to indicate the following regions of interest: promoter (2xOCS and 35S); intron (*adh1* intron I, *adh1* intron VI, and *pinII* intron I); gene (*cryIA(c)*, *bar* and *pinII*); 3' end (*pinII* and Tr7); f1 origin; ColE1 origin; and *bla* gene. The introns are shown as shaded arrows; the *pinII* genomic region (pDPG320) contains its own intron. Below each map are boxes which represent regions of interest for molecular analysis of DBT418 described in section V. Filled-in boxes indicate the hybridization probes produced by PCR and hatched boxes indicate the restriction fragments that are expected to hybridize with the PCR probes.

Table III.1. Summary of Sequences Present in Plasmid pDPG699

Vector Component	Size, Kb	Description
2XOCS:35S promoter	0.15	A chimeric promoter consisting of two copies of the OCS element (Bouchez et al., 1989), in reverse orientation, coupled to a 90-bp segment of the promoter for the 35S transcript from Cauliflower Mosaic Virus (Odell et al. 1985).
adh1 intron VI	0.42	The intron VI from the maize *adh1* gene (alcohol dehydrogenase-1; Dennis et al., 1984).
cryIA(c)	1.85	Synthetic DNA containing the protein-coding region for the first 613 amino acids of the HD73 CryIA(c) endotoxin from *Bacillus thuringiensis*.
Potato *pinII* 3'-end	0.93	A potato genomic DNA fragment from plasmid pRJ15 (C.A. Ryan, Washington State University). This DNA contains the last 10-bp of the potato pinII protein-coding region and ~920-bp of 3' sequence that includes both the 3' untranslated region and putative transcription termination region. (Thornburg et al., 1987).
lac	0.56	A partial lac repressor (lacI) coding sequence, the promoter plac, and a partial coding sequence for ß-galactosidase (lacZ). From the phagemid pBluescript SK[-] (Stratagene).
f1(-) ori	0.46	f1 bacteriophage origin of replication from the phagemid pBluescript SK[-] (Stratagene).
bla	0.86	The ß-lactamase gene (*bla*) is from the phagemid pBluescript SK[-] (Stratagene). This gene confers resistance on bacterial cells to ampicillin and other penicillins (Sutcliffe, 1978).
ColE1 ori	0.55	Plasmid origin of replication from the *E. coli* high copy phagemid pBluescript SK[-] (Stratagene).

Table III.2. Summary of Sequences Present in Plasmid pDPG165

Vector Component	Size Kb	Description
35S promoter	0.83	The Cauliflower Mosaic Virus (CaMV) promoter for the 35S transcript (Odell et al., 1985)
bar	0.55	The *bar* gene encoding phosphinothricin acetyltransferase isolated from *Streptomyces hygroscopicus* (White et al., 1990).
Tr7 3'-end	0.52	The untranslated 3' region from *Agrobacterium tumefaciens* T-DNA transcript 7 (Dhaese et al., 1983)
lac	0.56	A partial lac repressor (lacI)coding sequence, the promoter plac, and a partial coding sequence for b-galactosidase (lacZ) protein (Yanisch-Perron et al., 1985)
bla	0.86	The β-lactamase gene (*bla*) from *E. coli* plasmid pBR322 confers resistance on bacterial cells to ampicillin and other penicillins (Sutcliffe, 1978). The gene is under control of its native bacterial promoter.
ColE1-ori	0.55	The origin of DNA replication from the *E. coli* high copy plasmid pUC19 (Yanisch-Perron et al., 1985)

Table III.3. Summary of Sequences Present in Plasmid pDPG320

Vector Component	Size Kb	Description
35S promoter	0.43	The promoter for the 35S transcript from cauliflower mosaic virus (CaMV) (Odell et al. 1985).
adh1 intron I	0.57	The first intron from the maize gene *adh1*, (alcohol dehydrogenase-1) (Dennis et al., 1984).
Potato *pinII*	1.51	A potato genomic DNA fragment from plasmid pRJ15 (C.A. Ryan, Washington State University). Contains 18-bp of untranslated leader, the pinII protein-coding region with intron, and ~920-bp of 3' sequence (3' untranslated region of the RNA and the putative transcription termination region ; (Thornburg et al., 1987).
Tr7 3'-end	0.52	Untranslated 3' region from the transcript 7 gene from *Agrobacterium tumefaciens* T-DNA (Dhaese et al., 1983).
lac	0.56	A partial lac repressor (lacI) coding sequence, the promoter plac, and a partial coding sequence for beta-galactosidase (lacZ). This region is from the phagemid pBluescript SK[-] (Stratagene).
f1(-) ori	0.46	f1 bacteriophage origin of replication from phagemid pBluescript SK[-] (Stratagene); allows for recovery of the antisense strand of the lacZ gene when *E. coli* containing the phagemid are co-infected with helper phage.
bla	0.86	The β-lactamase gene (*bla*) from the phagemid pBluescript SK[-] (Stratagene). Confers resistance on bacterial cells to penicillins (Sutcliffe, 1978).
ColE1 ori	0.55	Origin of replication from the *E. coli* high copy phagemid pBluescript SK[-] (Stratagene).

IV. Open Reading Frames in pDPG165, pDPG699, and pDPG320

A. *cryIA(c)*

The *B.t.* gene that was used to produce the transgenic corn line DBT418 encodes a protein that is identical to the first 613 amino acids of a naturally occurring *Bacillus thuringiensis* subsp. *kurstaki* (also referred to as the HD73) CryIA(c) protein (Adang et al, 1985). The *cryIA(c)* gene in the corn line DBT418 consists of a DNA sequence that has been modified to contain an increased number of codons that are preferred for expression in maize. The DNA sequence of the *cryIA(c)* gene, and the corresponding amino acid sequence of the protein, are presented in Appendix I. CryIA(c) protein has been produced in *B.t.* microbial pesticide products (DIPEL®: Abbott Laboratories; MVP®: Mycogen, Inc.) that have been approved for use on a variety of crops with exemptions from the requirement for a tolerance.

B.t. proteins are very specific in their activity, exhibiting toxicity to a narrow range of insect species. Extensive reviews of *B.t.* proteins and their activities are available. In brief summary, microbially produced *B.t.* endotoxins of the *kurstaki* type are typically produced as "full-length" protoxin proteins of about 133,000 daltons (1,155 amino acids). These *B.t.* proteins form crystalline bodies either alone, or in combination with other *B.t.* proteins, that are eventually released from the microbe's mother cell. Upon ingestion by an insect, the alkaline conditions in many lepidopteran insect guts results in solubilization of the crystalline protein.

Subsequent proteolysis in the insect gut results in removal of about 29 amino acids from the amino-terminus and about 500 residues from the carboxy terminus of the protein. The result is a "processed" or truncated *B.t.* protein that is slightly more than 600 amino acid residues in length, but still contains the portion of the native protein that is responsible for insecticidal activity. In susceptible insects, the processed protein binds to specific receptors located on the lining of the gut. The interaction with these specific receptors results in the development of membrane pores that result in paralysis of the gut, leading to growth reduction and eventually death.

B. *bar*

The *bar* gene was originally isolated from *Streptomyces hygroscopicus* (ATCC 21705) and encodes phosphinothricin acetyltransferase (PAT). This enzyme is useful as a selectable marker as well as a source of resistance to the herbicide phosphinothricin (also known as glufosinate, the active ingredient in the herbicides Basta®, Rely®, Finale®, Liberty®). Phosphinothricin is a glutamic acid analog and is herbicidally active by inhibition of glutamine synthetase (Thompson et al.,1987).

PAT enzyme inactivates phosphinothricin by acetylation. The PAT enzyme is highly substrate specific (Thompson et al.,1987) with a substrate specificity for phosphinothricin over 300 times

higher than the affinity for the amino acid glutamate. PAT has no homology to any known toxins or allergens and has no known insecticidal activity.

C. *pinII*

In DNA preparations used to produce DBT418, the protease inhibitor II gene from potato (*pinII*) was also included. If produced at sufficient concentrations in plants, certain protease inhibitors have been shown to have insecticidal activity against specific insects (Ryan, 1990). Protease inhibitors form complexes with specific classes of proteases, thereby inhibiting proteolytic activity. As described below in section V., a single partial copy of *pinII* integrated in DBT418 and is non-functional.

D. *bla*

The *cryIA(c)*, *bar* and *pinII* vectors all contain the *E. coli bla* (ampicillin-resistance) gene. This gene encodes β-lactamase and is derived from the parent plasmid pUC19 (Yanisch-Peron et al., 1985) or pBluescript SK[-] (Stratagene). The β-lactamase protein cleaves and inactivates certain penicillin antibiotics. The gene expression elements regulating the *bla* gene in these plasmids provide for expression in certain bacterial hosts used to produce the plasmid DNA and it is highly unlikely that these expression elements will function in plants. This was demonstrated for DBT418 as described below in section VI.

V. Molecular and Genetic Analysis of DBT418 Corn

Summary

The DBT418 transformation event involved incorporation of plasmids containing the *cryIA(c)*, *bar*, and potato *pinII* genes into the maize genome (Stephens et al., 1996). Southern hybridization analyses were used to determined that hemizygous DBT418 plants contain approximately two intact copies of the *cryIA(c)* gene, approximately one intact copy of the *bar* gene, rearranged *bar* DNA at a level equivalent to one copy, and one partial rearranged copy of the *pinII* gene. In addition, DBT418 plants contain approximately four intact copies and one partial copy of the ampicillin resistance gene (*bla*) and approximately four intact copies of the ColE1 origin of replication. Four DBT418 backcross populations were analyzed by Polymerase Chain Reaction (PCR) for segregation of *cryIA(c)* and *bar* to determine if the DBT418 insertion segregated in Mendelian fashion (Walters, 1996). In all four populations, *cryIA(c)* and *bar* segregated together in plants at an approximately 1:1 ratio to plants lacking the transgenes. The genetic stability of the *cryIA(c)* gene in DBT418 plants was also determined by Southern hybridization analyses (Stephens et. al, 1996). Of 188 plants scored, 184 (97.9%) contained the expected genotypic pattern and four plants (2.1%) had lost one or both copies of *cryIA(c)*. This percentage of genotypic off-types is comparable to what is traditionally acceptable to the seed industry.

A. Transgene Content

Southern hybridization analyses were performed on genomic DNA from DBT418 plants to approximate the copy number of transgene sequences introduced by transformation (Figure V.1). The copy number of each transgene was estimated by comparison of hybridization intensity to a series of plasmid standards. In DBT418 plants there are approximately two copies of the *cryIA(c)*, two copies of the *bar* gene (one intact and one rearranged), and one incomplete copy of the potato *pinII* gene (Table V.1). The *cryIA(c)*, *bar* and *pinII* genes were introduced into corn cells on plasmids that contained the *bla* gene and the ColE1 origin of replication. Consistent with the above copy number estimates, approximately four copies of the *bla* gene and four copies of the ColE1 origin of replication were detected in DBT418 DNA (Table V.1).

The *pinII* expression vector, pDPG320, contains the maize *adh*1 intron I as an enhancer of gene expression. This intron also occurs in the endogenous copy of maize *adh*1 present in both nontransformed and transformed maize genomic DNA. Consequently, hybridization to this sequence provides a method to validate our plasmid DNA copy number standards. The *pinII* nucleic acid blot shown in Figure V.1 was stripped of the *pinII* probe, and rehybridized with a probe for the maize *adh*1 intron I (Figure V.2). As expected, this probe hybridized to a high molecular weight DNA in every lane, corresponding to the endogenous maize *adh*1 gene. This

gene is present in two copies in the maize diploid genome, confirming the accuracy of our plasmid DNA standards.

This experiment also demonstrated the presence of a single copy of the *adh*1 intron I sequence at another locus in DBT418 DNA (lanes 1-3), but not in the nontransformed control DNA (lane 4). This transgenic copy of *adh*1 intron I originated from plasmid pDPG320, since neither pDPG165 or pDPG699 contains this sequence.

For some of the probes used in this experiment, there were also examples of nonspecific hybridization to every DNA sample, including the nontransformed control DNA (Figure V.1). For example, the *pinII* probe hybridized very faintly to an approximately 5 kb restriction fragment in each lane. The *bar*, *bla* and ColE1 probes hybridized strongly to nucleic acid of less than 0.5 kb; we presume this was due to nonspecific hybridization to RNA. In addition, the *cryIA(c)* probe hybridized to a very high molecular weight band in every lane, with the intensity equivalent to one or less gene copies. Since these instances of hybridization occurred with non-transformed control DNA, we concluded that they represented nonspecific hybridization. Therefore, we did not include them in our estimates of gene copy number.

The results in Figure V.1 indicated that the *pinII* expression unit from pDPG320 had lost some sequence upon integration into the maize genome, i.e., either the *Xba*I or *Eco*RI restriction site, or both (see Figure III.1). To further characterize the nature of the deleted sequence, we attempted to amplify regions of the *pinII* coding region in DBT418 DNA by PCR, using nine different sets of primers (Figure V.3). A transgenic plant that contains the intact *pinII* gene was used as a non-DBT418 positive control. When either positive control plant DNA or pDPG320 DNA was amplified by PCR with the nine different primer sets, the expected PCR product was observed in each case (Figure V.3). However, amplification of DBT418 DNA with the same nine primer sets resulted in a PCR product from only one primer set, pin1 and pinG, and the PCR product was larger than expected indicating that the *pinII* coding region is rearranged These data indicate that for the partial *pinII* gene present in DBT418 DNA, at least nine different pairs of short sequence loci are no longer present in the positions required for generation of a functional *pinII* protein. Consequently, we conclude that an intact *pinII* coding region is not present in DBT418 plants.

B. Mendelian Segregation of *cryIA(c)* and *bar*

Four DBT418 genotypes were chosen for testing. Each of the genotypes represented efforts to introgress the DBT418 insertion event by backcrossing into inbred lines for eventual use in hybrid production. In each step of the backcrossing program, hemizygous transgenic plants are crossed with nontransgenic inbred plants. The progeny of each of these crosses would be expected to segregate 1:1 for the transgene insertion if the insertion is a single nuclear genetic locus.

PCR was performed on 43 DBT418 samples of each of the four genotypes and the single non-transformed control plant that was extracted with each set of 43 transgenic samples. DNA from the DBT418 and non-transformed control plants was analyzed for *cryIA(c)*, *bar*, and an endogenous maize gene, *adh*, that encodes alcohol dehydrogenase. Included in each set of PCR reactions for each DBT418 genotype were two positive control PCR reactions containing plasmid DNA, either pDPG165 or pDPG699, as the template. Two negative control reactions, containing no DNA, were included in each set of PCR reactions.

PCR analysis of the four DBT418 genotypes and non-transgenic control genotype is summarized in Tables V.2 and V.3. In all DBT418 genotypes tested, *cryIA(c)* and *bar* appear to segregate together in plants in approximately a 1:1 ratio to plants that lack the transgenes. This data includes the results from six plants that were re-extracted after appearing to contain *bar* and lack *cryIA(c)*, or contain *cryIA(c)* and lack *bar*. Upon re-testing, five of the six plants that were re-tested yielded results indicating either the presence or lack of both genes in each plant. The single plant of the genotype designated DBT418(AW)08(aBK) that appeared to contain *cryIA(c)* but lack *bar* may have been the result of a relatively rare recombination event. As would be expected, all non-transgenic control plants tested lacked the *cryIA(c)* and *bar* genes. Also, as expected, all of the plants tested, DBT418 or controls, were positive for the *adh* gene.

The data presented in Table V.3 demonstrate that the *cryIA(c)* and *bar* genes in DBT418 are tightly linked and segregate together in Mendelian fashion. All of the chi-square values obtained for segregation of *bar* and *cryIA(c)* in plants resulting from DBT418 backcrosses yielded P values greater than 0.05 (5% significance level). These data indicate that the *cryIA(c)* and *bar* genes in DBT418 are likely the result of the same insertion event and have been stably maintained in the corn genome through several generations of backcrossing.

C. Stability of the DBT418 Transgene Insertion

As described above, the DBT418 insertion event in DBT418 DNA was shown to be inherited in a Mendelian manner consistent with a single nuclear allele. As a further demonstration of stability, a large number of DBT418 plants were analyzed by Southern blot to determine the frequency at which variations in *cryIA(c)* gene content occurred. DNA from DBT418 plants was cleaved with a restriction enzyme which cleaves the *cryIA(c)* expression cassette at the single *Nco*I site at the start of the open reading frame coding for the CryIA(c) protein. Hybridization with *cryIA(c)* probe identified two *cryIA(c)* containing fragments of DNA 10-kb and 6.3-kb in length. This hybridization pattern has remained consistent from the original transformation event produced three years and several generations earlier; it was used as a diagnostic indicator of the DBT418 insertion event.

Non-segregating DEKALB hybrid seed, produced by crossing a female elite inbred (DK) homozygous for the DBT418 insertion with a non-transgenic male inbred (DL), were planted in the field and leaf tissue was collected from 190 of these plants. DNA was isolated from each plant and characterized by Southern hybridization analysis (Figure V.4). The DNA from two

samples did not give a clear signal due to incomplete digestion or degradation of the DNA and were not evaluated (see Figure V.4, lane 22, for example). Of the remaining 188 plants, 184 (97.9%) had the expected genotype for DBT418 and four plants (2.1%) did not (Table V.4). Three of these four plants contained a single copy of the *cryIA(c)* gene, present on the 6.3-kb *Nco*I fragment of DNA (see Figure V.4, lane 12). The other plant had neither copy of the *cryIA(c)* gene. It is possible that this plant lost both copies of the *cryIA(c)* gene due to a rearrangement involving repeated copies of plasmid DNA sequence at the transgene locus. A second possibility is that this individual resulted from fertilization with stray nontransgenic pollen in a previous generation. This level (2.1%) of genotypic off-types is within the limit of acceptability for the seed industry (Wych, et al., 1988).

Table V.1. DBT418 Copy Number Determination by Southern Blot Hybridization Analysis

Region Analyzed	Approximate Gene Copy Number		Was Gene Expression Detected?	Location of Molecular and Gene Expression Characterization in the Petition
	Intact	Rearranged		
cryIA(c)	2	0	yes	pgs. 22-23, 29, 33-34, 40-41
bar	1	1	yes	pgs. 22-23, 29, 35-36, 42-43
pinII	0	0.5	no	pgs. 22-23, 29, 30, 31, 36-37, 44, 45
*adh*1 intron I (*pinII*)[1]	0	0.5	not applicable	pgs. 22-23, 30
bla	4	0.5	no	pgs. 22-23, 29, 37-38, 44, 46
ColE1	4	0	not applicable	pgs. 22-23, 29

[1]The copy number of the *adh*1 intron I that is associated with the *pinII* gene was determined (the probe also identified the two endogenous gene copies of the maize *adh*1 gene).

Table V.2. Summary of PCR Analysis to Determine Segregation of the DBT418 Insertion

Genotype	Total No. Plants Tested	Gene Tested By PCR	No. PCR Positive Plants	No. PCR Negative Plants	χ^2	P
AW/BC5/DBT418	43	*cryIA(c)*	22	21	0	0.95
		bar	22	21	0	0.95
		adh	43	0	N/A	N/A
BS/BC5/(AW.DBT418)	43	*cryIA(c)*	24	19	0.37	0.50
		bar	24	19	0.37	0.50
		adh	43	0	N/A	N/A
DK/BC6/(AW.DBT418)	43	*cryIA(c)*	24	19	0.37	0.50
		bar	24	19	0.37	0.50
		adh	43	0	N/A	N/A
DBT418(AW)08(aBK)	43	*cryIA(c)*	20	23	0.10	0.70
		bar	19	24	0.37	0.50
		adh	43	0	N/A	N/A
AW	5	*cryIA(c)*	0	5	N/A	N/A
		bar	0	5	N/A	N/A
		adh	5	0	N/A	N/A

N/A = not applicable

Table V.3. Cosegregation of *cryIA(c)* and *bar* Genes in DBT418 Backcross Progeny Tested by PCR.

Genotype	*cryIA(c)* positive, *bar* positive	*cryIA(c)* positive, *bar* negative	*cryIA(c)* negative, *bar* positive	*cryIA(c)* negative, *bar* negative
AW/BC5/DBT418	22	0	0	21
BS/BC5/(AW.DBT418)	24	0	0	19
DK/BC6/(AW.DBT418)	24	0	0	19
DBT418(AW)08(aBK)	19	1	0	23

Table V.4. Southern Analysis of the Genetic Stability of DBT418.

no. of Plants	presence of *cryIA(c)* gene		genotype	% of total
	10-kb DNA	6.3-kb DNA		
184	+	+	DBT418	97.9
3	-	+	rearranged	1.6
1	-	-	rearranged or nontransformed	0.5
2*	N/A	N/A	not evaluated	N/A

* Not included in statistics:
1 sample DNA degraded
1 sample incomplete digestion

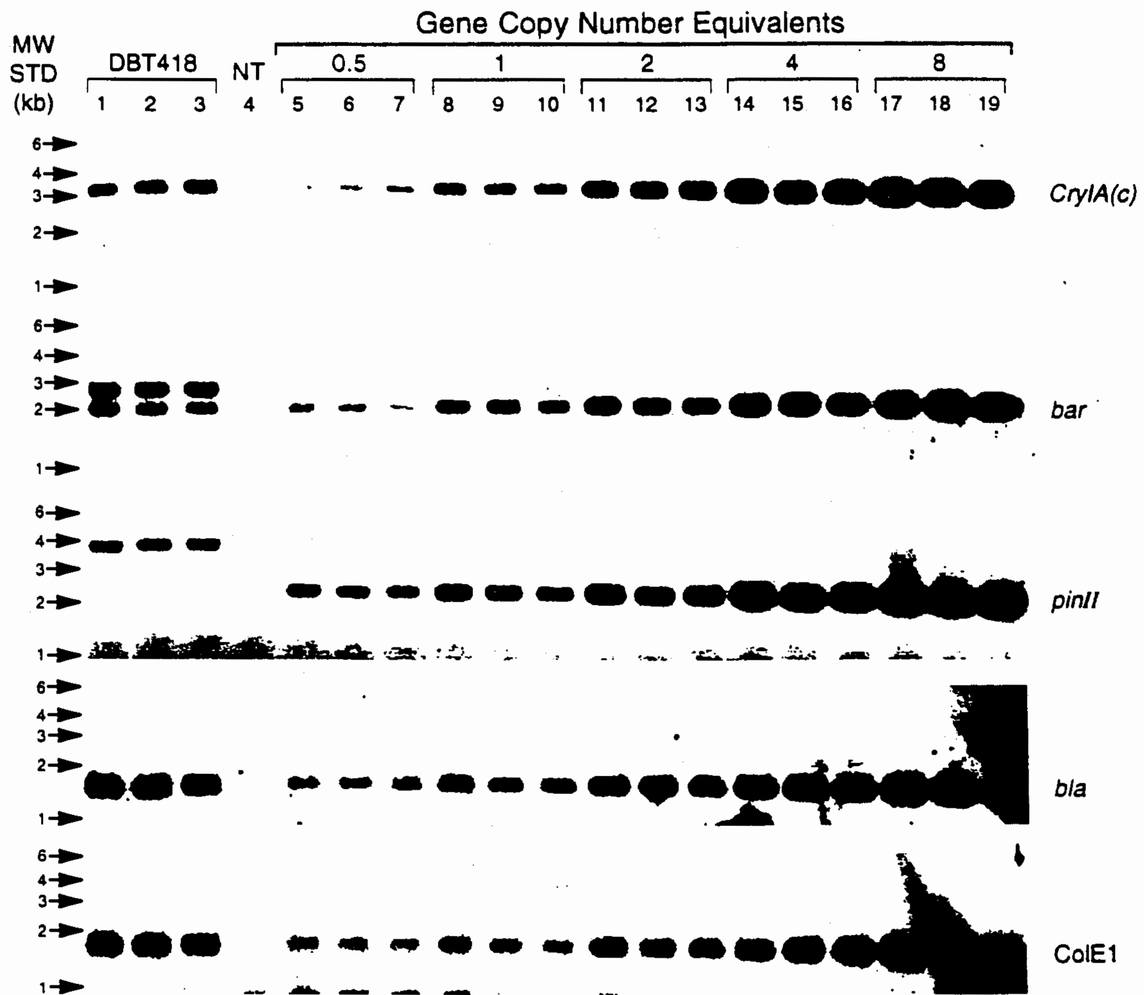


Figure V.1. Determination of Gene Copy Number Equivalents by Southern Hybridization. The PCR-generated probe used for each hybridization experiment is shown to the right (*cryIA(c)*; *bar*; *pinII*; *bla* and ColE1). Lanes 1-3: 10 µg of DBT418 DNA; lane 4: 10 µg of nontransformed (NT) DNA; lanes 5-19: 10 µg of nontransformed DNA with plasmid (pDPG699, pDPG165, or pDPG320) equivalent to 0.5,1 , 2, 4, or 8 gene copies, as indicated. The DNA in each panel was digested with the following restriction enzymes: *cryIA(c)*, *SalI/NotI*; *bar*, *Eco*RI/*Hin*dIII; *pinII*, *Eco*RI/*Xba*I; *bla*, *SspI/Afl*III; and ColE1,. *SspI/Afl*III. Molecular size standards (in kilobase pairs) are shown to the left.

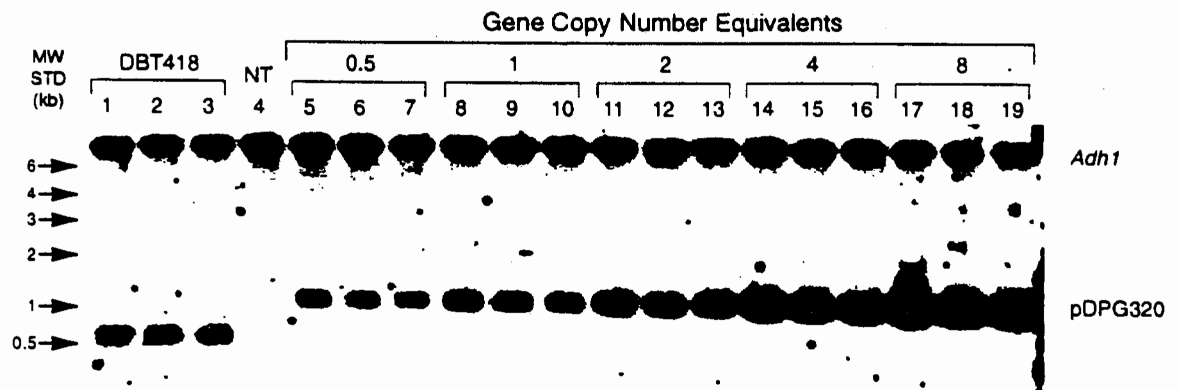
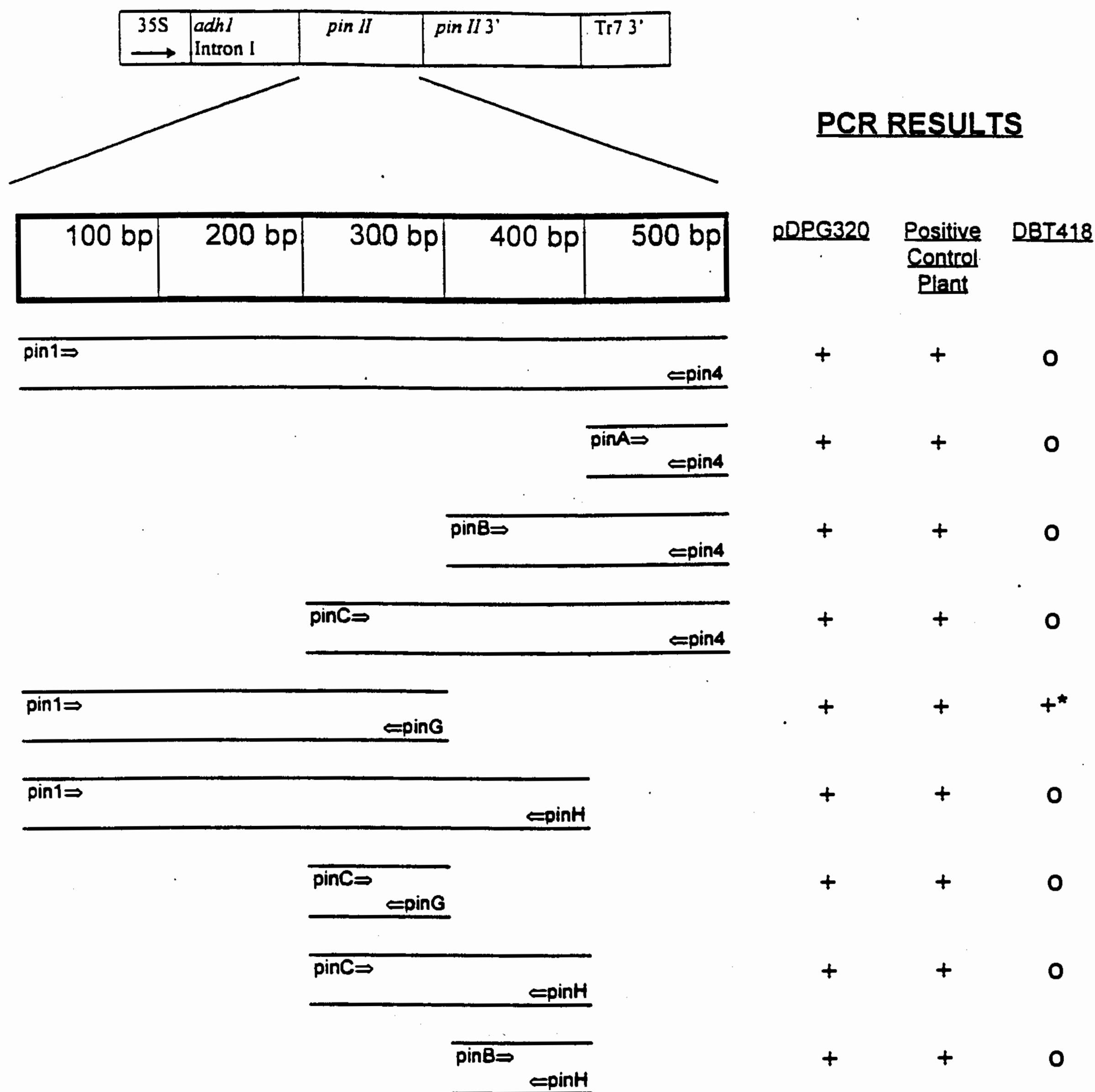


Figure V.2. Determination of Copy Number Equivalents of *adh1* Intron I Associated with the Potato *pinII* Gene. The Southern blot for *pinII* shown in Figure V.1 was heated to remove the *pinII* probe and rehybridized with a probe for *adh1* intron I.



* Expected size PCR product is ~300 bp. Positive control plant and pDPG320 displayed expected size PCR product. An ~500 bp product is produced from DBT418.

Figure V.3. **PCR Analysis of DBT418 DNA.** Top: Map of the *pinII* expression unit in pDPG320. Bottom, left: Positions of the nine different primer sets in the *pinII* coding region. Bottom, right: PCR amplification of three different DNAs with each of the nine primer sets resulted in either a detectable product of the expected size (+), a product of aberrant size (+*), or no detectable product (0). The positive control plant is a transformed maize line containing an intact copy of the potato *pinII* gene.

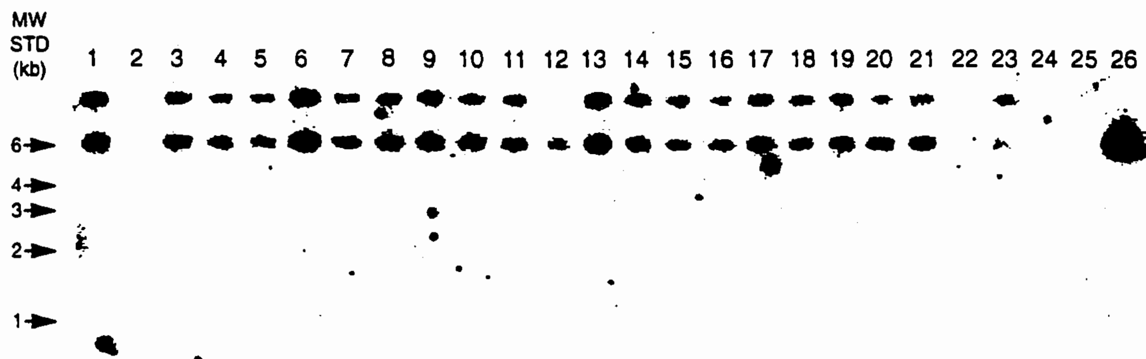


Figure V.4. **Stability of the *cryIA(c)* Gene in DBT418 Plants.** DBT418 plant leaf DNA was digested with *Nco*I and hybridized with *cryIA(c)* probe. Lanes 1-23: Each lane contains 5 μg of DNA from one of 23 different DBT418 plants; lane 24: No DNA; lane 25: 5 μg of nontransformed maize DNA; lane 26: 5 μg of nontransformed maize DNA and six gene copy equivalents of pDPG699. Molecular size standards are shown to the left.

VI. Expression of Transgenes in DBT418 Corn

Summary

Corn plants of three genotypic backgrounds, all containing the DBT418 insertion event, were grown in the field and sampled over the course of plant development. Tissue samples representing leaf, root, stalk, kernel, silk, pollen and whole plants were collected, where appropriate, at V6-V7 stage (seedling), pollen shed stage, dough stage, harvest stage and senescent stage and analyzed to determine the levels of CryIA(c) protein and herbicide resistance marker protein (phosphinothricin acetyltransferase; PAT) present (Kruger et al., 1996). Expression levels of CryIA(c) were similar for the three genotypic backgrounds analyzed. CryIA(c) protein was detected at maximum levels of 1198, 124, 125, 43, 111, and 147 ng/g dry weight in leaf, stalk, root, kernel, silk, and whole plants (including roots), respectively. PAT protein was detected at maximum levels of 1099, 136, 95, 6, 133, and 120 μg/g dry weight in leaf, stalk, root, kernel, silk, and whole plants (including roots), respectively. Both proteins were most highly expressed in leaf tissue with lower levels present in kernels and undetectable levels in pollen. CryIA(c) levels did not decline through the harvest sampling stage.

Coding sequences of two additional proteins, PIN II and β-lactamase (partial in the case of PIN II), were also introduced as components of the DBT418 insertion event. Although PIN II and β-lactamase proteins were not expected to be present in DBT418 plants, various tissues were analyzed for the presence of these two proteins. No evidence of PIN II or β-lactamase expression was detected in DBT418 plants (Kruger et. al, 1996).

A. CryIA(c) Expression

Table VI.1 describes the genotypic backgrounds containing the DBT418 insertion event and control genotypes that were analyzed in this study of DBT418 transgene expression. Tissue samples were collected from three field locations, two in Illinois and one in Connecticut. All samples were collected during the 1995 growing season. CryIA(c) levels in DBT418 plants were analyzed by ELISA (Kruger et al., 1996). Table VI.2 is a summary of the results of CryIA(c) analysis of DBT418 with the data presented on a dry weight basis. Table VI.3 presents the same data converted to ng of CryIA(c) per gram fresh weight. Conversion was based on percent moisture of each tissue.

The data in Tables VI.2 and VI.3 indicates that the pattern of expression of the CryIA(c) protein was similar in all three genotypes analyzed. Of the three genotypes analyzed, DK.DL(DBT418) is a commercial hybrid genotype hemizygous for the DBT418 insertion event while AW/BC2/DBT418. BS/BC1/DBT418(2BT) (also referred to as 2BT hybrid) is homozygous for the insertion event. The 2BT hybrid generally exhibited higher levels of CryIA(c) expression than the hemizygous hybrid (e.g., leaf samples at all sampling stages, stalk tissue at harvest

stage, all three root stages, etc.). An exception to this pattern was observed in kernel tissue where both lines produced similar levels of CryIA(c) protein.

The third genotype analyzed, the DBT418 S4 inbred, resulted from selfing hemizygous plants and therefore segregated for the DBT418 event. Non-DBT418 plants were eliminated from the sampling program for this genotype by herbicide selection. Therefore, one-third of the plants sampled for transgenic protein analysis would be expected to consist of DBT418 homozygous plants and two thirds of the plants would be expected to be hemizygous. Depending upon the actual number of copies of the DBT418 insertion in the plants sampled at each timepoint, the levels of transgenic protein accumulation in this line might be expected to fall somewhere between the levels observed for the completely hemizygous and completely homozygous genotypes as described above. The DBT418 S4 inbred was indeed somewhat variable in its CryIA(c) expression (Tables V.2 and V.3), in several instances producing the highest levels of any of the three genotypes (e.g., pollen shed leaf, pollen shed root, pollen shed whole plant), but in other cases the expression was somewhat lower and more similar to the hemizygous line (e.g., harvest leaf, harvest root).

The *cryIA(c)* gene in DBT418 is under control of a chimeric promoter, consisting of the 90 base pair A-domain from the 35S promoter from Cauliflower Mosaic Virus and two copies of the octopine synthase (OCS) enhancer from the T-DNA of *Agrobacterium tumefaciens* (Benfey and Chua, 1990; Bouchez et al. 1989). The OCS enhancer used in the DBT418 transformation event is known to promote expression of genes in most vegetative plant tissues (Bouchez et al. 1989). Table VI.2 shows that, compared to the other tissues, the highest levels of CryIA(c) protein were found in the leaves throughout the growing season. Comparing the different sampling timepoints, the highest levels were observed at harvest stage. Mean harvest stage leaf CryIA(c) levels ranged from 459.6 to 1198.4 ng/g dry wt for the three genotypes. Leaf tissue sampled at pollen shed stage ranged from a mean of 93.7 to 335.0 ng CryIA(c)/g dry wt for the three genotypes analyzed. In general the levels of CryIA(c) protein do not decline with plant maturity in any of the tissues analyzed. It is not known if the increasing CryIA(c) concentrations found in harvest stage tissues represent synthesis of additional CryIA(c) protein. The increase in CryIA(c) concentration may reflect a decline in the dry matter content of senescing tissues in which CryIA(c) is stable and not mobilized for ear development, as are many of the biochemical constituents of maize vegetative tissues during kernel maturation.

Levels of CryIA(c) in kernels were generally low but detectable at the time of harvest. Mean levels ranged from 36.0 to 42.8 ng CryIA(c)/g dry wt for the three genotypes. At all growth stages sampled root tissue contained relatively moderate levels of CryIA(c), for example, harvest stage means ranged from 58.0 to 125.4 ng CryIA(c)/g dry wt for the three genotypes. These values are similar to the seedling stage and pollen shed stage expression levels.

CryIA(c) protein in silk tissue was below the level of detection of the assay in most cases and at detectable but moderate levels in two of the DK.DL(DBT418) samples analyzed (CryIA(c) limit of detection in silk was 51.6 ng/g dry wt.). CryIA(c) levels in stalk tissue were also low to moderate (40.9 to 123.6 ng CryIA(c)/g dry wt at harvest stage) for the three genotypes, however, it is expected that most of the CryIA(c) protein present in the stalk is located in the green tissue,

or associated with vascular tissue, rather than the more abundant pithy core. Analysis of the entire stalk, as performed here, would tend to underestimate the levels of CryIA(c) protein present in the stalk surface tissues to which ECB larvae would be initially subjected.

CryIA(c) protein was not detected in pollen. The limit of detection of the assay as performed on pollen tissue in this study is approximately 6.7 ng/g dry wt. This lack of pollen expression was supported by the results described by Walters and Kruger (1996) in which neonate tobacco hornworm (THW) larvae were reared on DBT418 pollen. DBT418 pollen had no observable toxic effect on larvae of the THW, which are highly sensitive to CryIA(c).

CryIA(c) concentration in senescent tissue was similar to that found in pollen shed stage whole plants. The senescent stage sample consisted of whole plants removed from the ground (including roots) at harvest time. Plants were allowed to remain in mesh bags on the ground surface for one month before the entire plant was pulverized, sub-sampled and analyzed for transgene protein expression.

B. PAT Expression

The same DBT418 genotypes, tissues, and growth stages that were analyzed for CryIA(c) levels were also analyzed for PAT levels. PAT quantification in DBT418 plants was performed using Western blots (Kruger et al., 1996). Table VI.4 presents a complete summary of the results of PAT protein quantification in DBT418 plants of a dry weight basis. Table VI.5 presents the same data presented on a fresh weight basis. The tissue distribution pattern for PAT expression was similar to that found for the CryIA(c) protein except that levels of PAT were significantly higher. The highest levels of PAT expression in DBT418 plants were found in leaves with means for the three genotypes ranging from 501.8 to 1099.4 μg/g dry wt at pollen shed stage. Unlike CryIA(c), however, PAT levels declined somewhat in the senescing tissues of harvest stage leaves, dropping to less than 25% of peak pollen shed stage levels, with harvest values ranging from 60.8 to 213.6 μg PAT/g dry wt. PAT levels in stalk, silk and root tissue were less than that of the leaf but still relatively high in comparison to CryIA(c). Pollen shed stage stalk mean amounts ranged from 60.0 to 77.0 μg PAT/g dry wt; root ranged from 27.5 to 69.5 μg PAT/g dry wt and silk expression levels ranged from 29.1 to 133.3 μg PAT/g dry wt. Relatively low levels of PAT protein were found in the kernel (range of 3.1 to 6.0 μg PAT/g dry wt for the three genotypes) and PAT expression in pollen was not detected. The limit of detection for PAT in pollen was 12.1 μg/g dry wt.

Comparison of PAT expression in the three different genetic backgrounds reveals some differences among the three genotypes, similar to the situation described for the CryIA(c) protein. As described, the AW/BC2/DBT418. BS/BC1/DBT418(2BT) line is homozygous for the DBT418 event while DK.DL(DBT418) is hemizygous. In many of the tissues, the PAT levels found in the line homozygous for the DBT418 insert were approximately double the levels found in the hemizygous genotype (e.g., all stages of leaf, harvest-stalk, pollen shed-root, pollen

A non-transgenic tissue, without spike, was also analyzed as a negative control. By verifying the ability of the assay to detect PIN II in the spiked tissue samples, the effectiveness of the complete procedure was validated and the sensitivity of the assay confirmed each time the analysis was performed.

Table VI.6 summarizes the sample set analyzed for PIN II expression. The three genotypes analyzed were in all cases the same genotypes analyzed for PAT and CryIA(c) expression, for all tissues except pollen. Pollen analysis was conducted on genotype AW/BC2/DBT418.BS/BC1/DBT418(1BT) because of a shortage of AW/BC2/DBT418 S4 pollen. Plants grown in two different field locations were analyzed for each Tissue X Growth Stage Combination.

An example of a PIN II Western blot is presented in Figure VI.1. In the PIN II Western blot detection assay, the same dry weight of tissue and volume of extraction buffer were used for each sample that was extracted to normalize protein loads. There is a significant amount of cross-reactivity of the primary and/or secondary antibodies that cause a number of higher molecular weight proteins in the extracts to be detected. However, the region of the gel at which the PIN II protein runs (approximately 12,300 daltons) is reasonably free from background in all tissues with the exception of pollen.

The limit of detection for the PIN II Western blot assay for most tissues (leaf, stalk, root) is 400 ng/g dry weight of tissue. Lower amounts of PIN II protein would be detectable in most instances since it should be possible to visually detect a fainter band than the one produced by the spiked sample. A PIN II protein band representing perhaps one half or less of the defined LOD would produce a visible band in the majority of the Western blots performed. There is no evidence for the presence of PIN II protein in any of the tissues analyzed. Comparison of control lanes with DBT418 lanes revealed no additional immunoreactive bands in the DBT418 extracts that were not present in the control extracts. Pollen tissue analysis was more problematic than the others due to the presence of cross-reactive proteins of about the same molecular weight as the PIN II protein. Because of the cross-reactive band in pollen it was impossible to determine with certainty the Limit of Detection of PIN II protein in pollen although it was possible in some instances to distinguish between the non-transgenic pollen protein background and the PIN II spikes. Comparison of transgenic and non-transgenic Western blots of all tissues analyzed revealed no differences. There were no immunoreactive protein bands present in the transgenic tissues, other than non-specific background bands also observed in the control plants.

D. Lack of β-Lactamase Expression

The β-lactamase gene (*bla*) in the plasmids used for creation of the DBT418 transformation event is present only as a selectable marker for the production of plasmid DNA in bacterial hosts and does not contain elements to promote expression of the gene in plants. It would not be expected, therefore, that the β-lactamase protein would be expressed in DBT418 plants. To confirm this expectation, immunoblot analysis for β-lactamase protein was conducted to determine the presence or absence of the protein in a variety of DBT418 plant tissues.

shed-whole plant and kernel). This would be expected if expression levels are additive based on the number of copies of the transgene insert present.

The third line analyzed, AW/BC2/DBT418 S4 segregates for the DBT418 event. Since the non-DBT418 plants have been eliminated by herbicide selection, one-third of the plants sampled for transgene protein analysis would be expected to consist of DBT418 homozygous plants and two-thirds of the plants would be expected to be hemizygous. As described for CryIA(c), depending upon the genotypic composition of the plants sampled, protein accumulation in the DBT418 S4 inbred might be expected to fall somewhere between the levels observed for the completely hemizygous and completely homozygous genotypes just described. This is true in some instances but not in all tissue/growth stages analyzed (i.e., harvest leaf, V6-V7 root, kernel, senescent whole plants and dough stage whole plants). In general, the plant genotypic background per se does not appear to exert a great deal of influence on PAT expression levels, with all genotypes examined expressing similar protein levels characteristic for a given tissue type.

PAT levels found in senescent plant tissue had declined from the peaks observed at the pollen shedding stage. This sample consisted of whole plants left to lie in mesh bags on the ground surface for one month before the entire plant was analyzed for transgene protein expression. PAT levels in the senescent tissue ranged from 12.1 to 28.6 μg PAT/g dry wt for the three genotypes.

C. Lack of PIN II Expression

One of the plasmids (pDPG320) used in the creation of the DBT418 insertion event contains the potato *pinII* gene. The PIN II protein is an inhibitor of serine proteases and has been shown to inhibit both trypsin and chymotrypsin (Ryan, 1990).

Molecular analysis of the DBT418 insertion event demonstrated that the *pinII* gene is not present in an intact form in the plant genome (section V.). The DBT418 event involved integration of only a portion of the PIN II coding sequence into the plant genome. Based upon this information alone it would be extremely improbable that the PIN II protein would be present in DBT418 plants. However, analysis for the presence of PIN II protein was conducted as a qualitative assay to determine the presence or absence of the protein in a variety of DBT418 plant tissues.

PINII protein levels in various DBT418 tissues were investigated using Western blots (Kruger et al., 1996). A PIN II protein enrichment procedure that was employed in this analysis to increase the sensitivity of the analysis beyond that achievable through direct analysis of crude protein extracts. The Limit of Detection (LOD) of the assay is based upon the ability to recover and detect PIN II from lyophilized non-transgenic control tissue samples spiked with the PIN II reference protein. In each set of samples extracted, fractionated and analyzed, a minimum of two control (non-transgenic) tissue samples of the same type and growth stage were spiked with the PIN II reference protein and processed in exactly the same fashion as the transgenic test samples.

Crude protein extracts were analyzed for the presence of β-lactamase by Western blot (Kruger et al., 1996). The Limit of Detection (LOD) of the assay is based upon the ability to recover and detect β-lactamase from lyophilized non-transgenic control tissue samples spiked with the reference protein. In each set of samples extracted and analyzed, a minimum of two control (non-transgenic) tissue samples of the same type and growth stage were spiked with the β-lactamase reference protein and processed in exactly the same fashion as the transgenic test samples. An unspiked non-transgenic extract was also included in each analysis as a negative control. By verifying the ability of the assay to detect β-lactamase in the spiked tissue samples, the effectiveness of the complete procedure was validated and the sensitivity of the assay confirmed each time the analysis was performed.

Table VI.7 summarizes the sample set analyzed for β-lactamase expression. The three genotypes analyzed were the same genotypes analyzed for PAT and CryIA(c) content. As described above for PINII protein analysis, pollen production by AW/BC2/DBT418.BS/BC1/DBT418 (2BT) was inadequate for analysis; pollen from another DBT418 genotype was substituted for β-lactamase analysis. Plants grown in two different field locations were analyzed for each Tissue X Growth Stage Combination.

An example of a β-lactamase Western blot is presented in Figure VI.2. In the β-lactamase Western blot detection assay, the same dry weight of tissue and volume of extraction buffer were used for each sample that was extracted to normalize total protein loads. Some cross reactivity of the primary and secondary antibodies with other protein bands was apparent with DBT418 and control protein extracts. However, the cross-reactivity is minimal and the region of the gel where the β-lactamase protein is found (approximately 32,000 daltons) is reasonably free from background in all tissues.

The limits of detection for the β-lactamase Western blot assay for all the tissues analyzed was less than 9 μg per gram dry weight of tissue in all cases. Lesser amounts of β-lactamase protein than this defined limit of detection would in fact be detectable (in some instances much less) on all of the blots performed, since a fainter band than the one produced by the spiked sample would be visible. Comparison of control lanes with DBT418 lanes revealed no additional immunoreactive bands in the transgenic plant extracts that were not present in the control extracts, either at the molecular weight expected for β-lactamase or at any other molecular weight range on the gel.

To summarize, there was no evidence for the presence of the β-lactamase protein in any of the tissues analyzed. Comparison of control lanes with DBT418 lanes revealed no additional immunoreactive bands in the DBT418 extracts that were not present in the control extracts. β-lactamase Western blot data supports the conclusion that there is no expression of the β-lactamase protein in DBT418 plants.

Table VI.1. Genotypic Backgrounds Used for Protein Level Determinations

Genotype	Abbreviation	DBT418 Allele	Description
AW/BC2/DBT418 S4	S4 inbred	segregating	unfinished DBT418 inbred
AW/BC2/DBT418. BS/BC1/DBT418(2BT)	2BT hybrid	homozygous	unfinished DBT418 hybrid
DK.DL(DBT418)	DK.DL(DBT418)	hemizygous	finished DBT418 hybrid
DK.DL	DK.DL	none	control hybrid
AW	AW	none	control inbred

note: Pollen analysis was performed on pollen from AW/BC2/DBT418.BS/BC1/DBT418(1Bt) in place of AW/BC2/DBT418 S4 and on inbred DK in place of AW.

Table VI.2. CryIA(c) Protein Levels on a ng/g Dry Weight Basis During DBT418 Maize Development

		Mean ng CryIA(c)/g dry weight (n; SE)				
Tissue	**Genotype**	**A** V6-V7	**B** POLLEN SHED	**C** DOUGH	**D** HARVEST	**E** SENESCENCE
leaf	AW/BC2/DBT418 S4	217.9 (7; 46.23)	335.0 (8; 74.93)	N/A	459.6 (8; 99.84)	N/A
	DK.DL(DBT418)	177.8 (8; 42.22)	93.7 (8; 7.39)	N/A	620.6 (8; 84.39)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	289.6 (4; 36.74)	174.2 (4; 36.69)	N/A	1198.4 (4; 270.78)	N/A
stalk	AW/BC2/DBT418 S4	N/A	28.5[c] (3; 4.19)	N/A	123.6[a] (7; 46.72)	N/A
	DK.DL(DBT418)	N/A	**BLD** (8)	N/A	40.9 (8; 7.34)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	115.1 (4; 25.19)	N/A
root ball	AW/BC2/DBT418 S4	69.8 (7; 17.34)	78.2 (8; 12.56)	N/A	58.7 (8; 10.16)	N/A
	DK.DL(DBT418)	50.9 (8; 9.58)	57.7[a] (7; 16.24)	N/A	58.0[b] (5; 8.22)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	117.9 (4; 17.08)	72.0 (4; 20.56)	N/A	125.4 (4; 16.93)	N/A
kernel	AW/BC2/DBT418 S4	N/A	N/A	N/A	42.8 (6; 16.60)	N/A
	DK.DL(DBT418)	N/A	N/A	N/A	37.1 (8; 3.97)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	N/A	36.0 (4; 8.14)	N/A
silk	AW/BC2/DBT418 S4	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	110.5[d] (2; 10.70)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	N/A	N/A
pollen	AW/BC2/DBT418.BS/BC1/DBT418(1BT) (+)	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	**BLD** (4)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (8)	N/A	N/A	N/A
whole plant with root ball	AW/BC2/DBT418 S4	N/A	147.1 (8; 47.87)	N/A	N/A	124.2 (8; 16.47)
	DK.DL(DBT418)	N/A	35.9 (8; 5.44)	N/A	N/A	41.2[a] (7; 6.42)
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	75.0 (4; 15.03)	N/A	N/A	69.9 (4; 17.76)
whole plant without root ball	AW/BC2/DBT418 S4	N/A	N/A	288.5 (8; 31.63)	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	57.6[a] (7; 9.65)	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	176.5 (4; 53.94)	N/A	N/A

BLD = Below Limit of Detection. Statistics presented are based on samples falling above the assay limit of detection. N/A = not applicable.
a = 1 of 8 samples were BLD, b = 3 of 8 samples were BLD, c = 5 of 8 samples were BLD, d = 6 of 8 samples BLD.
(+) = Genotype was substituted for AW/BC2/BT418 S4 since sufficient pollen was not available.

Table VI.3. CryIA(c) Protein Levels on a ng/g Fresh Weight Basis During DBT418 Maize Development

		Mean ng CryIA(c)/g fresh weight (n; SE)				
Tissue	**Genotype**	**A** V6-V7	**B** POLLEN SHED	**C** DOUGH	**D** HARVEST	**E** SENESCENCE
leaf	AW/BC2/DBT418 S4	33.6 (7; 7.12)	88.1 (8; 19.7)	N/A	240.4 (8; 52.22)	N/A
	DK.DL(DBT418)	27.4 (8; 6.50)	24.6 (8; 1.94)	N/A	324.6 (8; 44.14)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	44.6 (4; 5.66)	45.8 (4; 9.65)	N/A	626.8 (4; 141.62)	N/A
stalk	AW/BC2/DBT418 S4	N/A	5.7[c] (3; 0.84)	N/A	36.7[a] (7; 13.88)	N/A
	DK.DL(DBT418)	N/A	**BLD** (8)	N/A	12.1 (8; 2.18)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	34.2 (4; 7.48)	N/A
root ball	AW/BC2/DBT418 S4	7.0 (7; 1.75)	11.1 (8; 1.78)	N/A	10.8 (8; 1.87)	N/A
	DK.DL(DBT418)	5.1 (8; 0.97)	8.2[a] (7; 2.31)	N/A	10.7[b] (5; 1.51)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	11.9 (4; 1.72)	10.2 (4; 2.92)	N/A	23.1 (4; 3.12)	N/A
kernel	AW/BC2/DBT418 S4	N/A	N/A	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	N/A	N/A	N/A
silk	AW/BC2/DBT418 S4	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	14.1[d] (2; 1.37)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	N/A	N/A
pollen	AW/BC2/DBT418.BS/BC1/DBT418(1BT)[(+)]	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	**BLD** (4)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (8)	N/A	N/A	N/A
whole plant with root ball	AW/BC2/DBT418 S4	N/A	27.6 (8; 9.00)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	6.7 (8; 1.02)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	14.1 (4; 2.82)	N/A	N/A	N/A
whole plant without root ball	AW/BC2/DBT418 S4	N/A	N/A	97.2 (8; 10.66)	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	19.4[a] (7; 3.25)	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	59.5 (4; 18.18)	N/A	N/A

BLD = Below Limit of Detection. Statistics presented are based on samples falling above the assay limit of detection. N/A = not applicable.
a = 1 of 8 samples were BLD, b = 3 of 8 samples were BLD, c = 5 of 8 samples were BLD, d = 6 of 8 samples BLD.
(+) = Genotype was substituted for AW/BC2/BT418 S4 since sufficient pollen was not available.

Table VI.4. PAT Protein Levels on a μg/g Dry Weight Basis During DBT418 Maize Development

		Mean μg PAT/g dry weight (n; SE)				
Tissue	**Genotype**	**A** V6-V7	**B** POLLEN SHED	**C** DOUGH	**D** HARVEST	**E** SENESCENCE
leaf	AW/BC2/DBT418 S4	351.1 (7; 52.91)	522.0 (6; 59.04)	N/A	60.8[a] (6; 12.46)	N/A
	DK.DL(DBT418)	276.3 (8; 25.51)	501.8 (8; 34.75)	N/A	108.5 (8; 24.68)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	554.9 (2; 136.03)	1099.4 (3; 76.29)	N/A	213.6 (4; 61.92)	N/A
stalk	AW/BC2/DBT418 S4	N/A	75.8 (8; 12.24)	N/A	95.2 (6; 16.86)	N/A
	DK.DL(DBT418)	N/A	60.0 (8; 11.98)	N/A	64.4 (8; 8.23)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	77.0 (4; 11.66)	N/A	136.3 (2; 12.74)	N/A
root ball	AW/BC2/DBT418 S4	95.1 (7; 16.91)	54.1 (8; 9.15)	N/A	24.5 (7; 3.71)	N/A
	DK.DL(DBT418)	59.4 (8; 3.53)	27.5 (8; 6.25)	N/A	21.3 (8; 2.23)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	88.1 (4; 21.45)	69.5 (4; 23.58)	N/A	28.8 (3; 7.37)	N/A
kernel	AW/BC2/DBT418 S4	N/A	N/A	N/A	6.0 (6; 1.88)	N/A
	DK.DL(DBT418)	N/A	N/A	N/A	3.1 (8; 0.35)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	N/A	4.9 (4; 0.63)	N/A
silk	AW/BC2/DBT418 S4	N/A	128.2 (8; 17.21)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	29.1 (8; 2.97)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	133.3 (2; 60.01)	N/A	N/A	N/A
pollen	AW/BC2/DBT418.BS/BC1/DBT418(1BT)[(+)]	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	**BLD** (8)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	N/A	N/A
whole plant with root ball	AW/BC2/DBT418 S4	N/A	111.1 (8; 16.50)	N/A	N/A	28.6 (8; 3.09)
	DK.DL(DBT418)	N/A	72.8 (8; 5.88)	N/A	N/A	12.1 (6; 3.53)
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	119.5 (4; 25.63)	N/A	N/A	20.8 (3; 5.80)
whole plant without root ball	AW/BC2/DBT418 S4	N/A	N/A	190.5 (8; 30.76)	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	39.5 (7; 7.51)	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	135.2 (3; 10.42)	N/A	N/A

BLD = Below Limit of Detection. Statistics presented are based on samples falling above the assay limit of detection. N/A = Not Applicable.

a = 2 of 8 samples analyzed were BLD and were not used to calculate the mean or SE.

(+) = Genotype was substituted for AW/BC2/BT418 S4 since sufficient pollen was not available.

Table VI.5. PAT Protein Levels on a μg/g Fresh Weight Basis During DBT418 Maize Development

Tissue	Genotype	Mean μg PAT/g fresh weight (n; SE)				
		A V6-V7	**B** POLLEN SHED	**C** DOUGH	**D** HARVEST	**E** SENESCENCE
leaf	AW/BC2/DBT418 S4	54.1 (7; 8.15)	137.3 (6; 15.53)	N/A	31.8[a] (6; 6.52)	N/A
	DK.DL(DBT418)	42.6 (8; 3.93)	132.0 (8; 9.2)	N/A	56.7 (8; 12.91)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	85.4 (2; 20.95)	289.1 (3; 20.06)	N/A	111.7 (4; 32.38)	N/A
stalk	AW/BC2/DBT418 S4	N/A	15.2 (8; 2.45)	N/A	28.3 (6; 5.00)	N/A
	DK.DL(DBT418)	N/A	12.0 (8; 2.40)	N/A	19.1 (8; 2.44)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	15.4 (4; 2.33)	N/A	40.5 (2; 3.78)	N/A
root ball	AW/BC2/DBT418 S4	9.6 (7; 1.71)	7.7 (8; 1.30)	N/A	4.5 (7; 0. 68)	N/A
	DK.DL(DBT418)	6.0 (8; 0.36)	3.9 (8; 0.89)	N/A	3.9 (8; 0.41)	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	8.9 (4; 2.17)	9.9 (4; 3.35)	N/A	5.3 (3; 1.36)	N/A
kernel	AW/BC2/DBT418 S4	N/A	N/A	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	N/A	N/A	N/A
silk	AW/BC2/DBT418 S4	N/A	16.4 (8; 2.20)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	3.7 (8; 0.38)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	17.1 (2; 7.68)	N/A	N/A	N/A
pollen	AW/BC2/DBT418.BS/BC1/DBT418(1BT)(+)	N/A	**BLD** (8)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	**BLD** (8)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	**BLD** (4)	N/A	N/A	N/A
whole plant with root ball	AW/BC2/DBT418 S4	N/A	20.9 (8; 3.10)	N/A	N/A	N/A
	DK.DL(DBT418)	N/A	13.7 (8; 1.10)	N/A	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	22.5 (4; 4.82)	N/A	N/A	N/A
whole plant without root ball	AW/BC2/DBT418 S4	N/A	N/A	64.2 (8; 10.37)	N/A	N/A
	DK.DL(DBT418)	N/A	N/A	13.3 (7; 2.53)	N/A	N/A
	AW/BC2/DBT418.BS/BC1/DBT418(2BT)	N/A	N/A	45.6 (3; 3.51)	N/A	N/A

BLD = Below Limit of Detection. Statistics presented are based on samples falling above the assay limit of detection. N/A = Not Applicable.
a = 2 of 8 samples analyzed were BLD and were not used to calculate the mean or SE.
(+) = Genotype was substituted for AW/BC2/BT418 S4 since sufficient pollen was not available.

Table VI.6. Summary of Western Blot Analysis for PIN II in DBT418 Tissues

Tissue	Growth Stage	# of Genotypes Evaluated	Total # of DBT418 Plants Analyzed	Assay Limit of Detection (per g dry wt.)	PIN II Detection
Leaf	V6-V7	3	10	400 ng	ND
Leaf	Pollen Shed	3	10	400 ng	ND
Stalk	Pollen Shed	3	6	400 ng	ND
Root	Pollen Shed	3	6	400 ng	ND
Pollen	Pollen Shed	3	6	*	ND
Kernel	Harvest	3	10	1800 ng	ND

*Limit of detection for pollen was not accurately determined. See discussion.
ND = Not Detected

Table VI.7. Summary of Western blot analysis of DBT418 tissues for β-lactamase protein.

Tissue	Growth Stage	# of Genotypes Evaluated	Total # of DBT418 Plants Analyzed	Assay Limit of Detection (per g dry wt.)	β-lactamase Detection
Leaf	V6-V7	3	11	9 μg	ND
Leaf	Pollen Shed	3	10	9 μg	ND
Stalk	Pollen Shed	3	5	9 μg	ND
Root	Pollen Shed	3	5	9 μg	ND
Pollen	Pollen Shed	3	5	9 μg	ND
Kernel	Harvest	3	10	9 μg	ND

ND = Not Detected

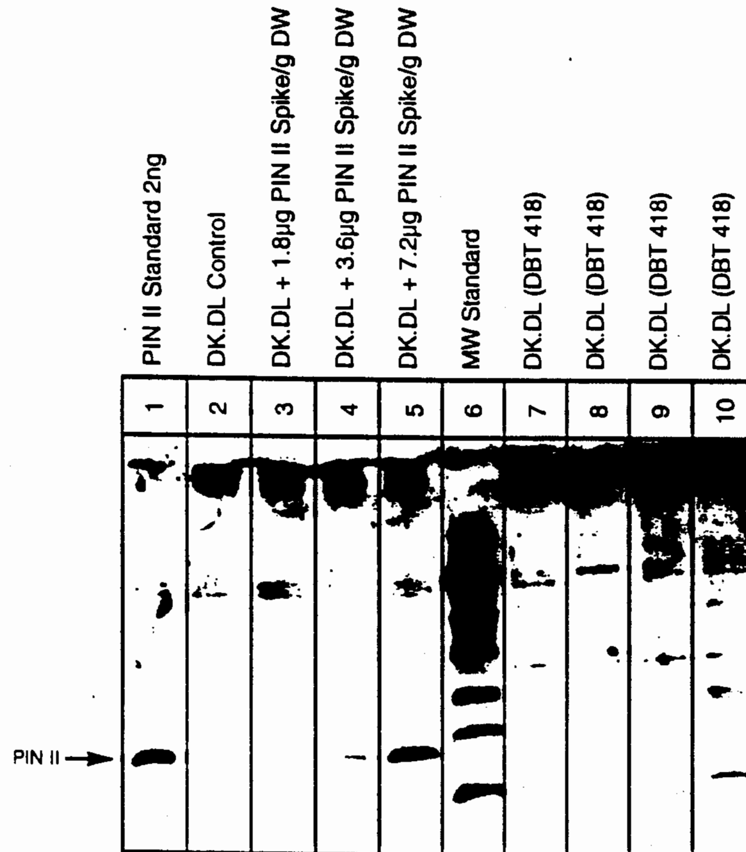


Figure VI.1. Western blot of PIN II in kernel extracts. PINII reference standard is shown in lane 1. Lane 2 is an enriched extract from a non-transgenic control plant. PINII protein band is evident in enriched extracts of spiked DK.DL analyzed in lanes 3, 4, and 5 (DW = dry weight). No PINII band is visible in enriched kernel extracts of four different DBT418 plants in lanes 7-10.

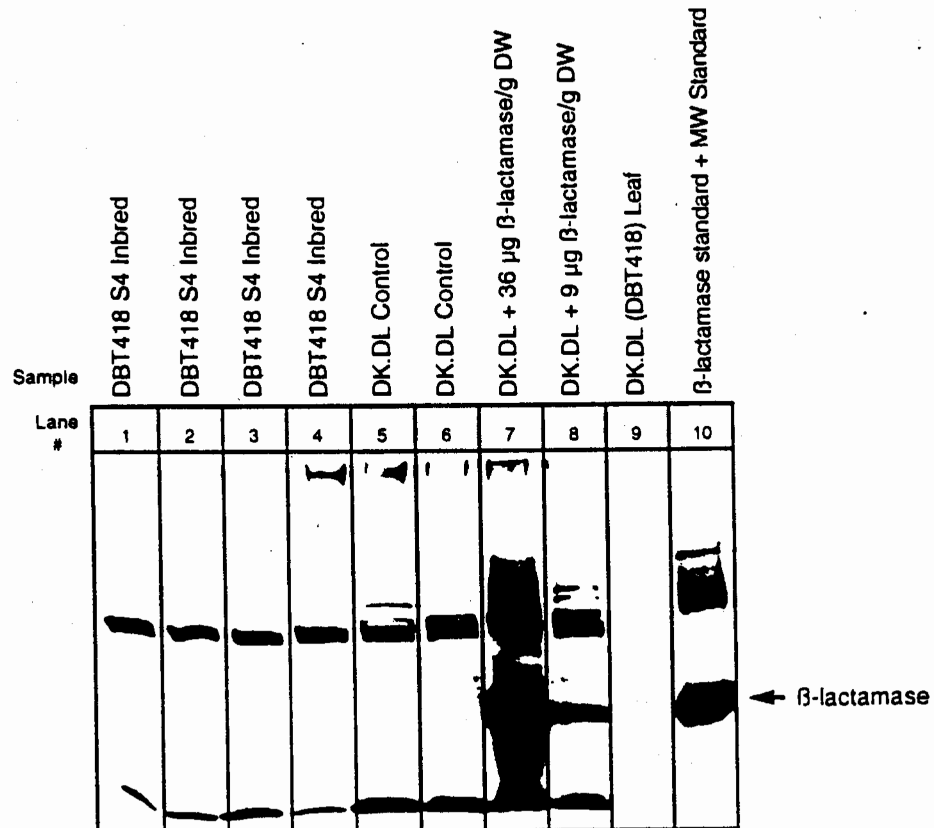


Figure VI.2. Western blot of β-lactamase in kernel extracts. β-lactamase reference standard is shown in lane 10. Lanes 5 and 6 are extracts from non-transgenic control plants. β-lactamase protein band is evident in extracts of spiked DK.DL analyzed in lanes 7 and 8 (DW = dry weight). No β-lactamase band is visible in kernel extracts of four different DBT418 S4 inbred plants in lanes 1-4. Lane 9 is an extract of DK.DL(DBT(418) leaf with no β-lactamase evident.

VII. Field Trials and Compositional Analysis of Insect Protected Maize Line DBT418

A. Field Trials

Insect protected maize line DBT418 has been field tested in the U.S. by DEKALB under APHIS notifications since 1993. DBT418 USDA/APHIS field release notifications from 1993 to 1996 are listed below in Table VII.1. DBT418 field tests between 1993 and 1996 were performed in 20 U.S. states as well as in France, Italy, Argentina and Canada.

Three types of field tests have been conducted in the United States. First, early tests were designed to evaluate several transformant sources and the level of insect resistance present in each one. Second, efforts have continued since 1993 to introgress the insect and glufosinate resistance traits into a wide spectrum of DEKALB elite inbreds. Third, in 1995 and 1996, finished DBT418 hybrids were evaluated for agronomic performance.

1. Agronomic Performance

Agronomic performance evaluation of a DEKALB hybrid containing the DBT418 insertion event, as compared to the unconverted version of the same hybrid, is presented in Table VII.2. Traits evaluated included yield, grain moisture, final stand count, seedling vigor, plant height, pollen GDU (growing degree units), silk GDU, stay-green, intactness, % dropped ears, % stalk lodging, % root lodging, and % barren plants. Plants were evaluated from ten locations grown in 1995, two reps per location. In general, the performance of the DBT418-converted hybrid was very similar to the unconverted version of the same hybrid. Slight differences, significant at the $P = 0.5$ level, were detected between the unconverted and converted version of the hybrid as relates to grain moisture, seedling vigor, silk GDU's, staygreen, and intactness. However, as shown in Table VII.2, the magnitude of these differences was very small.

2. Disease and Pest Characteristics

Insect protected maize line DBT418 has been field tested in the United States since 1993, as well as in France, Italy, Argentina and Canada. In the process of field testing, transgenic lines are evaluated as non-transgenic lines would be by a corn breeder, i.e., the breeder visually inspects for symptoms such as leaf spotting or streaking, wilting, leaf necrosis, leaf yellowing or other visible symptoms of insect or disease damage. Field trials are conducted at multiple locations in a single field season and conducted over multiple years. Multiple location, multiple year field testing has been used successfully by plant breeders to identify disease or insect susceptibilities in new hybrids.

DEKALB routinely monitors field locations for disease and insect pests. Helminthosporium leaf spot (*Helminthosporium carbonum*), common corn rust (*Puccinia sorghi*), Stewart's disease (*Erwinia stewartii*), common corn smut (*Ustilago maydis*), anthracnose (*Colletotrichum graminicolum*), southern corn leaf blight (*Helminthorsporium maydis*), northern corn leaf blight (*Helminthosporium turcicum*), Helminthosporium leaf disease (*Helminthosporium rostratum*), eyespot (*Kabatiella zeae*), southern corn rust (*Puccinia polysora*), zonate leaf spot (*Gloeocercospora sorghi*), gray leaf spot (*Cercosporo zeae-maydis*), and a variety of ear and stalk rots were identified at locations where DBT418 was field released (see Appendix III for particular diseases present at each field release location). Although these diseases were identified at DBT418 field release locations, the severity of each disease varied from year to year and location to location and depends on environmental conditions. In no instance was any difference in disease susceptibility observed between the DBT418 line and counterpart nontransgenic lines.

Insect populations of European corn borer (*Ostrinia nubilalis*), Corn rootworm (*Diabrotica* spp.), Southwestern corn borer (*Diatraea crambidoides*), Fall armyworm (*Laphygma frugiperda*), Corn earworm (*Heliothis zea*), and various other insect pests were observed at DBT418 field locations (see Appendix III for distribution of insect pests at release locations). At most locations the primary insect pests were Corn rootworm and European corn borer. Differences observed in the susceptibility of the DBT418 line and counterpart nontransgenic lines were control of European corn borer, significant protection from Southwestern corn borer, and growth inhibition of Corn earworm by DBT418. DBT418 may also have some impact on Lesser cornstalk borer and Sugarcane borer, however these insects were only present at a very limited number of release locations and the impact of DBT418 on these species has not been fully evaluated. Southwestern corn borer, Sugarcane borer, and Lesser cornstalk borer are limited to the Southern U.S. and will receive little or no exposure to DBT418 depending on the geographic distribution of the product.

B. Compositional Analysis

Corn is the most important crop in American agriculture. Corn is used by three major consuming groups: livestock, industrial, and human. In 1980-1985, 83% of the corn crop was used for animal feed for hogs, cattle and dairy, and poultry. Wet-millers produce starch, feed, syrup, oil, and dextrins. Corn can be dry-milled to produce corn meal, flour, grits, oil and breakfast cereals. In addition, the distilling and fermentation industries in the United States produce ethyl and butyl alcohol, acetone, and whiskey. Corn products enter many human foods including bakery and dairy products, beverages, and confections. Industrial uses include paper products, construction materials, textiles, metal castings, pharmaceuticals, ceramics, paints, explosives and many others.

The major component of corn grain is starch which constitutes about 73% of the mature kernel, 98% of which is in the endosperm. The kernel contains about 9% total protein and about 74% of the protein is present in the endosperm. The other major constituent of the corn kernel is the fat or oil which is primarily localized in the embryo, about 83% of the fat or oil is found in the embryo.

Compositional analysis was performed on grain harvested from a hybrid containing the DBT418 insertion event and a nontransgenic version of the same hybrid. The levels of the major

components of the maize grain were determined by Woodson-Tenent Laboratories (Des Moines. IA) using conventional chemical methods (Association of Official Analytical Chemists, AOAC; and American Oil Chemists Society, AOCS) and by DEKALB using Near Infrared Transmission Spectroscopy (NIT). These data are summarized in Tables VII.3 and VII.4. Chemical determination of compositional analysis was performed on samples collected from ten locations grown in the U.S. in 1995. NIT data was derived from samples collected from nine locations grown in the U.S. in 1995. Composition of grain from the DBT418 hybrid was essentially identical to that of the conventional hybrid. A slight difference in protein levels was observed using chemical methods (Table VII.3) however this difference was not observed using NIT (Table VII.4).

Table VII.1. DBT418 USDA/APHIS Notifications for Field Releases in the U.S.

APHIS Notification No.	Location
93-127-03N	DeKalb Co., IL; Maui Co., HI
94-081-03N	Maui Co., HI
94-081-06N	New London Co., CT; Champaign Co., DeKalb Co., IL; Clay Co., NE; Obion Co., TN
94-115-02N	McLean Co., Ogle Co., IL
94-220-02N	McLean Co., IL; Maui Co., HI; Palm Beach Co., FL
94-243-01N	Dade Co., FL
95-010-02N	Beauford Co., Wayne Co., NC:
95-020-04N	Maui Co., HI
95-072-25N	New London Co., CT; Lee Co., GA; DeKalb, Vermillion, Sangamon , Mason, Champaign Counties, IL; Daviess, Tipton Counties, IN; Cass, Webster, Johnson , Clay Counties, IA; Haskell Co., KS; Ingham Co., MI; Clay, Renville, Steele Counties, MN; Saline Co., MO; Adams Co., NE; Marion Co., OH; Lancaster Co., PA; Minnehaha Co., SD; Obion, Weakley Counties, TN; Columbia Co., WI
95-072-26N	New London Co., CT; DeKalb, Tazewell Counties, IL; Renville Co., MN; Obion Co., TN
95-111-07N	Dixson Co., NE
95-138-02N	Maui Co., HI
95-221-02N	Dade Co., FL; Maui Co., HI
95-228-04N	Dade Co., FL; Kauai Co., HI
96-023-01N	Hildago Co., TX
96-029-01N	Tift Co., GA
96-054-10N	New London Co., CT; Lee Co., GA; Haskell Co., KS; Buchanan, Holt, Saline Counties, MO; Wayne Co., NC; Lancaster Co., PA; Minnehaha Co., SD; Obion , Weakley Counties, TN; Columbia Co., WI.
96-054-11N	Clay, Jackson, Olmstead, Renville, Steele Counties, MN.
96-054-12N	Kauai, Maui Counties, HI.
96-054-13N	Dade Co., FL
96-054-14N	Blackhawk, Boone, Buena Vista, Cass, Clay, Dickinson, Fayette, Grunde, Humboldt, Johnson, Marshall, Powesheik, Story, Webster Counties, IA.
96-054-15N	Champaign, DeKalb, Douglas, Lee, Logan, Macon, Mason, Sangamon, Tazewell, Vermillion, Warren, Whiteside Counties, IL.
96-054-16N	Ingram, St. Joseph Counties, MI.
96-054-17N	Adams, Buffalo, Burt, Clay, Dawon, Gospur, Kearney, Saunders, York Counties, NE.
96-054-18N	Poro Nergo [sic] Division, PR.
96-054-19N	Benton, Daviess, Jasper, LaPorte, Montgomery, Tippecanoe, Tipton Counties, IN.
96-054-20N	Marion Co., OH.
96-081-01N	LaSalle Co., IL, Polk, Greene Counties, IA
96-086-08N	Sussex, Kent Counties, DE
96-099-01N	Knox Co., MO
96-099-10N	Prince Georges Co., MD

Table VII.2. Agronomic Performance of a DBT418-Converted Hybrid as Compared to the Conventional Version of the Same Hybrid.

trait	counterpart unconverted hybrid	DBT418
yield (bushels/acre)	130.4	129.5
grain moisture (%)	13.9	14.3[a]
test weight (lbs.)	55.0	55.0
final stand count	61.2	61.1
seedling vigor (1-9 scale)	6.5	6.2[a]
plant height (in.)	89.2	88.5
ear height (in.)	42.5	41.0
pollen GDU	1339	1342
silk GDU	1335	1342[a]
stay-green (1-9 scale)	4.2	4.3[a]
intactness (1-9 scale)	4.1	4.9[a]
dropped ears (%)	0.04	0.04
stalk lodged (%)	3.1	2.9
root lodged (%)	2.9	5.0
barren plants (%)	3.4	5.1

[a] statistically different from the control at the P = 0.5 level

Table VII.3. Proximate Analysis of Grain from DBT418 Plants Performed Using Chemical Methods

Characteristic	DBT418 ± S.E. (N)	Control ± S.E. (N)	Literature Range
Protein	9.02 ± 0.22 (30)	8.56 ± 0.16 (30)	6.0-12.0[a]
Oil	4.05 ± 0.05 (30)	3.92 ± 0.04 (30)	3.1-5.7[a]
Fiber	1.96 ± 0.03 (30)	2.02 ± 0.03 (30)	2.0-5.5
Ash	1.32 ± 0.01 (30)	1.30 ± 0.02 (30)	1.1-3.9[a]
Moisture	8.14 ± 0.04 (30)	8.22 ± 0.04 (30)	7-23[a]

[a] Watson, 1987

Table VII.4. Proximate Analysis of Grain from DBT418 Plants Performed Using NIT

Characteristic	DBT418 ± S.E. (N)	Control ± S.E. (N)	Literature Range
Protein	9.06 ± 0.19 (27)	9.09 ± 0.17 (27)	6.0-12.0[a]
Oil	4.16 ± 0.04 (27)	4.12 ± 0.04 (27)	3.1-5.7[a]
Starch	70.61 ± 0.20 (27)	70.62 ± 0.17 (27)	
Moisture	5.63[b] ± 0.17 (27)	5.57[b] ± 0.11 (27)	7-23[a]

[a] Watson, 1987
[b] samples were artificially dried

VIII. Environmental Consequences of the Introduction of DBT418

A. Effects on Non-target Organisms

1. *Lack of Pollen-Based Exposure*

The activity of CryIA(c) protein in DBT418 pollen was evaluated using a tobacco hornworm (THW) bioassay (Walters and Kruger, 1996). As indicated above (section VI.A), the level of CryIA(c) protein in DBT418 pollen was undetectable using ELISA methodology. THW was chosen as a test organism given that it is extremely sensitive to CryIA(c) protein. The assays were performed by incorporating fresh or freeze-dried pollen into an agarose matrix. Pollen was incorporated into the agarose matrix at a concentration of 0.25 g pollen/ml agarose. The pollen and agarose served as the only food source for THW for a period of five days. No toxic effects, mortality or growth inhibition, were observed over multiple repetitions of the five-day assay. These data indicate that CryIA(c) expression in DBT418 pollen is so low as to present no risk of toxicity to non-lepidopterous insect species such as the honeybee or other non-target insects or to fish that may be exposed to DBT418 pollen.

2. *Exposure to Non-target, Beneficial Insects*

There is extensive literature describing the specificity of microbial preparations of *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) proteins which include CryIA(c) protein. *B.t.k.* proteins have been shown to be very selective for lepidopteran insects (MacIntosh et al., 1990; Aronson et al., 1986; Whitely and Schnepf, 1986; Klausner, 1984; Dulmage, 1981), bind specifically to the mid-gut of lepidopteran insects (Van Rie et al., 1990; Van Rie et al., 1989; Hofmann et al., 1988a; Hofmann et al., 1988b; Wolfersberger et al., 1986), and have no deleterious effects on beneficial insects (Melin and Cozzi, 1989; Vinson, 1989; EPA, 1988; Flexner et al., 1986; Krieg and Langenbruch, 1981; Cantwell et al., 1972).

The CryIA(c) protein produced in Monsanto's Bollgard™ Cotton is 99.4% identical (based on amino acid sequence) to the protein produced by the *B.t.k.* HD73 bacterial strain, and is similar in immunoreactivity and molecular weight to CryIA(c) contained in the microbial insecticides Dipel® and Thuricide® (Serdy et al., 1994). The insecticidally-active, tryptic core of the Monsanto CryIA(c) protein is approximately 99% identical (differing by six out of approximately 600 amino acids) to the tryptic core of the native *B.t.k.* CryIA(c) protein. The Monsanto, full length, *E. coli* produced CryIA(c) protein was demonstrated to be functionally equivalent to the tryptic fragment of native *B.t.k.* CryIA(c) when tested in bioassays against ten insect species representing five different orders (Sims, 1994). As described in section IV.A, the CryIA(c) protein produced in DBT418 is identical to the first 613 amino acids of the *B.t.k.* CryIA(c) protein, which includes the trypic core. The CryIA(c) protein in DBT418 plants was shown to be nearly identical to the native CryIA(c) protein produced in *Bacillus thuringiensis*,

based on molecular weight, immunogenicity, amino-terminal sequence, insecticidal activity, and glycosylation (Millham et al., 1996).

Monsanto has demonstrated the lack of effect of an *E. coli* produced CryIA(c) protein (identical to that produced in Bollgard^{TM} cotton) on honey bee larvae and adults, lady beetle, green lacewing, and parasitic wasp. Honey bee larvae and adults were exposed to 20 ppm CryIA(c) protein; no toxicity was observed (Maggi 1993a; Maggi 1993b). Honey bee larval studies were terminated after emergence of adults; toxicity tests with adult bees were terminated after seven days. Lady beetles were exposed to 20 ppm CryIA(c) protein in diet for 30 days, no toxicity was observed (Palmer and Beavers, 1993a). Green lacewing larvae were exposed to 20 ppm CryIA(c) protein over a period of 11 days, no toxicity was observed (Palmer and Beavers, 1993b). Parasitic Hymenoptera were exposed to 20 ppm CryIA(c) protein over a period of 23 days, no toxicity was observed (Palmer and Beavers, 1993c). The exposure levels in the studies described above greatly exceed any potential exposure to CryIA(c) based on exposure to DBT418 pollen. No CryIA(c) was detected in DBT418 pollen with a detection limit of 6.7 ppb (based on dry weight). In the insect toxicity studies described above, the maximum CryIA(c) concentration was greater than 2985 times the maximum concentration of CryIA(c) in DBT418 pollen. In addition, the maximum CryIA(c) concentration tested was also greater than 16 times the maximal level of CryIA(c) found in DBT418 leaves (1.2 ppm; based on dry weight). These results demonstrate the lack of toxicity of CryIA(c) protein to a range of non-target insects.

3. *Exposure to Birds*

An avian toxicity study was performed to assess the potential toxicity of corn containing CryIA(c) protein to birds (Palmer and Beavers, 1996). The test was performed using young quail and lyophilized DBT418 leaf tissue incorporated at 20% w/w (200,000 ppm) into diet. Leaf tissue was chosen as the test substance due to the fact that CryIA(c) protein levels were demonstrated to be the highest in leaves (section VI.A). No mortalities or overt signs of toxicity were observed for any of the test or control birds. All birds were normal in appearance and behavior throughout the 5-day test period and 3-day observation period. No treatment-related effects on body weight or feed consumption were observed. The no observed effect level for lyophilized DBT418 leaf was considered to be greater than 20% w/w.

4. *Exposure to Soil Organisms*

A collembola chronic toxicity test was performed given the potential exposure to collembola via incorporation of DBT418 plant material into the soil (Collins, 1996). Two test substances were chosen for the test: lyophilized DBT418 leaf tissue and microbially-produced CryIA(c) protein. Leaf tissue was chosen as a test substance to most accurately represent exposure to collembola in the field. Two concentrations of lyophilized leaf were tested: 2 and 8 g/kg soil. Microbially-produced CryIA(c) was also used for the test to provide exposure levels that greatly exceed typical exposure in the field. CryIA(c) was tested at 0.1 mg/kg soil. The collembola toxicity study was conducted as two separate experiments (Tables VIII.1 and VIII.2). After the 28-day exposure period, survival of the test organisms was reduced in the DBT418 leaf treatments and

the corresponding nontransformed control leaf treatments, as compared to the untreated control. However, no difference in survival was observed between DBT418 leaf treatments and the corresponding nontransformed control leaf treatments. No differences between offspring production were observed between the test substances (DBT418 leaf or microbially produced CryIA(c)), control substances, or the untreated controls.

Table VIII.1. Collembola toxicity study, experiment 1.

Treatment	mean percent survival[b]	mean number of cumulative offspring produced
2.0 g DBT418 lyophilized leaf/kg art. soil	65[a]	616
0.1 mg microbially-produced CryIA(c)/kg art. soil	78	398
2.0 g control lyophilized leaf/kg art. soil	55[a]	380
0.1 mg attenuated microbially-produced CryIA(c)/kg art. soil	68[a]	596
untreated control	100	439

[a] significantly different from untreated control
[b] no collembola were rated as lethargic in any treatment

Table VIII.2. Collembola toxicity study, experiment 2.

Treatment	mean percent survival[b]	mean number of cumulative offspring produced
8.0 g DBT418 lyophilized leaf/kg art. soil	53[a]	234
8.0 g control lyophilized leaf/kg art. soil	48[a]	152
untreated control	95	215

[a] significantly different from untreated control
[b] no collembola were rated as lethargic in any treatment

An earthworm toxicity test was performed given the potential exposure of earthworms to CryIA(c) protein via incorporation of DBT418 plant material into the soil (Garvey, 1996). As in the collembola study described above, two test substances were chosen for the test: lyophilized DBT418 leaf tissue and microbially-produced CryIA(c) protein. Test substance treatments consisted of two different concentrations of DBT418 leaf tissue (2 and 8 g/kg soil) and one combination of DBT418 leaf tissue and microbially-produced CryIA(c) protein (2 g leaf + 0.1 mg CryIA(c)/kg soil). No effect on earthworm mortality was observed at any of the test

substance concentrations for the 14-day test period. No difference in weight gain was observed between test substance treatments and corresponding control treatments.

5. Exposure to Mammals

To assess the acute toxicity of CryIA(c) protein to mammals, ten mice, five males and five females, were administered a dose of 5000 mg of microbially produced CryIA(c) preparation/kg body weight by oral gavage. The test substance consisted of the insecticidally active, tryptic core of CryIA(c) protein purified from a fermentation of *Bacillus thuringiensis* subsp. *kurstaki* strain no. 35866 (ATCC). Production and characterization of the microbial CryIA(c) protein is described in Merriman (1996a). The equivalence of the microbially-produced CryIA(c) used in this study to CryIA(c) protein produced in DBT418 plants was demonstrated in Millham, et al., 1996). The protein preparation administered to mice in this study was shown to be 66.5% CryIA(c) on a weight basis; therefore the mice received a dose of 3325 mg of CryIA(c) protein/kg body weight. No acute oral toxicity of the CryIA(c) protein to mice was observed during the 14-day test period. Based on the results of the test, the acute oral LD_{50} was estimated to be greater than 5000 mg of the CryIA(c) preparation/kg body weight. Based on 66.5% purity of the test substance, this corresponds to an LD_{50} of greater than 3325 mg of CryIA(c) protein/kg body weight.

The acute toxicity of PAT protein was assessed using histidine-tagged PAT (His-tag PAT) protein (purified from a fermentation of *E. coli*) administered to mice. Histidine-tagging (Novagen) the PAT protein provided a workable means of purifying gram quantities of PAT protein necessary for the mouse toxicity study. Production and characterization of His-tag PAT protein is described in Merriman (1996b). The functional equivalence of the microbially-produced, His-tag PAT used in this study to PAT protein produced in DBT418 plants was demonstrated in Lacetti et al. (1996). For the PAT acute toxicity study, ten mice, five males and five females, were administered a dose of 2500 mg of the His-tag PAT preparation/kg body weight by oral gavage (Merriman, 1996b). The protein preparation administered to mice in this study was shown to be 100% His-tag PAT on a weight basis. No acute toxicity was observed during the 14-day test period. Based on the results of the test, the acute oral LD_{50} was estimated to be greater than 2500 mg of His-tag PAT/kg body weight.

As part of the mammalian safety assessment, CryIA(c) and PAT proteins were tested for digestibility using simulated gastric fluid and Western blot detection (Walters and Adams, 1996). Both proteins were found to rapidly degrade in full strength and dilute simulated gastric fluid. CryIA(c) degraded to below detection limits after a few seconds in full strength (1X) simulated gastric fluid. In simulated gastric fluid in which the pepsin concentration had been reduced 100-fold (0.01X), CryIA(c) degraded to below detection in five minutes. PAT degraded to trace levels after 2 minutes in full strength (1X) simulated gastric fluid. No PAT was detectable after 5 minutes in 0.01X simulated gastric fluid. The rapid degradation of CryIA(c) and PAT proteins supports the safety of these proteins for mammalian consumption.

6. Impact on Endangered Species

No endangered of threatened lepidopteran insects, as listed in 50 CFR 17.11 and 17.12, feed on corn plants.

B. Environmental Fate

Transgenic corn plants that produce pesticidal proteins have the potential to release these proteins into the soil environment, particularly when the corn plant is left on, or incorporated into the soil either after harvest or earlier in the growing season. Reports in the scientific literature (Palm et al., 1994; Pruett et al., 1980; West et al., 1984) demonstrate rapid loss of insecticidal activity following incorporation of *Bacillus thuringiensis* subsp. *kurstaki* proteins into soil.

DEKALB has tested the effect of CryIA(c) protein on two soil-inhabiting organisms: collembola and earthworms (Collins, 1996, Garvey, 1996). In these studies, test organisms were exposed to CryIA(c) levels in vast excess of the likely typical environmental exposure resulting from CryIA(c) corn. No toxicity to collembola or earthworms was observed as the result of exposure to CryIA(c) protein.

To assess the environmental fate of DBT418 CryIA(c) protein, the amount of CryIA(c) protein per acre of DBT418 corn was calculated (Stephens, 1996). Approximately 1.0 g/acre represents the maximal amount of CryIA(c) that would be incorporated into the soil following harvest. This calculation, along with literature and studies described above, indicate it is highly unlikely that CryIA(c) incorporation into the soil, in the form of DBT418 plant tissue, will have any significant impact on nontarget organisms.

C. The Herbicide Glufosinate, Herbicide Usage in Corn, Impact on Current Practices, and Likelihood of Appearance of Glufosinate Resistant Weeds

As mentioned above, DEKALB's glufosinate resistant corn line B16 was granted nonregulated status by USDA/APHIS on December 19, 1996. The insect protected maize line DBT418 was created using the same *bar* plasmid that was used to create B16. In the petition submitted by DEKALB requesting nonregulated status for B16 (petition 95-145-01), a detailed discussion of the herbicide glufosinate, herbicide usage on corn, impact on current practices, and likelihood of appearance of glufosinate resistant weeds was presented.

D. Weediness Potential of the Line DBT418

1. Outcrossing to Wild Zea Species

Zea mays can only cross with species of the genus *Tripsacum* or *Zea*. Although *Zea mays* has been hybridized to species of the genus *Tripsacum* under experimental conditions, the progeny are usually sterile (Galinat, 1988; Mangelsdorf, 1974; Russell and Hallaner, 1980). Therefore, only outcrossing to *Zea* species will be considered here.

Both perennial and annual species of *Zea* are found in Mexico and Guatemala. However, no wild species of *Zea* grow in the United States. Although corn and teosinte species are cross fertile, the species are reproductively isolated by flowering time, geographic distribution and block inheritance. Although none of these factors insures complete reproductive isolation, it is highly unlikely that the hybrid would survive outside cultivation or be selected for further cultivation. No cases of gene flow between corn and its wild relatives are known in the United States.

2. Outcrossing to Cultivated Zea Varieties

Corn is an open pollinated crop. Therefore there is a high probability of cross pollination of corn plants in adjacent fields if they are sexually receptive at the time of pollen shed. Purity of seed production fields is maintained by isolation by at least 660 feet from adjacent pollen shedding fields, temporal isolation (no synchrony between pollen shed and female receptivity), or in some instances by the use of non-harvested border rows or natural barriers to pollen dispersal. These isolation techniques are used to control outcrossing to adjacent corn fields.

The grain harvested from experimental and commercial hybrids is consumed by animals. Farmers and breeders do not save open pollinated seed of hybrids for future crop plantings as this seed will not be true to the hybrid type. If the insect or glufosinate resistance trait were transferred to another variety of corn via open cross pollination, the resulting grain will be destroyed by animal consumption and therefore not perpetuated in a breeding population or corn production. Therefore, it is highly unlikely that the traits would be transferred to and maintained in other varieties of corn via open cross pollination. In any case, if a chance pollination of a second variety occurred, corn does not usually establish as a volunteer weed in other crops and when present can be controlled before flowering by a variety of agronomic practices including cultivation and/or chemical weed control.

3. Weediness or Pest Potential of Insect protected Corn

It is unlikely that genetically engineered corn will become a weed. Baker (1974) developed a list of characteristics that would be possessed by the ideal weed. Keeler (1989) examined these characteristics with relationship to genetically engineered plants. The fundamental question to be addressed was whether it is likely that insertion of a DNA sequence is likely to alter a crop species in such a way that it will directly become a weed or contribute to the weediness of another species. Whereas most weeds have seven or more of the described characteristics, corn has three. If each of these characteristics were controlled by a single gene, it would be necessary to alter four or more genetic loci to render corn a weed. It is unlikely that multiple genetic modifications would occur following insertion of a single gene into a plant through genetic engineering techniques as would be necessary to confer weedy characteristics on corn. Furthermore, the presence of these characteristics in corn would reduce the value of corn as a cultivated crop. Weed characteristics such as discontinuous germination and continuous seed production would be detrimental to economically efficient cultivation of corn. High seed production characteristic of many weed species is absent from corn. The modern corn hybrid does not compete well in the environment, as is evidenced by the dramatic decreases in yield under conditions where there is competition from weeds. Furthermore, corn seed does not readily disperse in the environment, as the seed is large and the seed bearing ear is enclosed in the husk. Through centuries of domestication and breeding corn has developed as a crop that is dependent on human intervention for its cultivation. Corn cannot survive without human intervention, and is not capable of surviving as a weed (Galinat, 1988; Rissler and Mellon, 1993).

E. Potential for Gene Transfer to Other Organisms

It is highly unlikely that transgenes will be transferred from DBT418 corn to other organisms that are not sexually compatible with corn if the plants are released into the environment. In the Environmental Assessment prepared by USDA-APHIS prior to approval of APHIS permit number 90-332-02 for the first field release of the genetically modified glufosinate resistant line B16 by DEKALB, it was stated, "Horizontal movement of the introduced genes is not possible. No mechanism for horizontal movement is known to exist in nature to move an inserted gene from a chromosome of a transformed plant to any other organism" (USDA-APHIS, 1991). Furthermore, USDA-APHIS has consistently maintained in determinations of deregulated status for transgenic crops, beginning with the determination of non-regulated status for the Calgene FLAVR SAVR™ tomato (USDA-APHIS, 1992), that, "There is no published evidence for the existence of any mechanism, other than sexual crossing," for the transfer of a transgene from a plant to a sexually incompatible organism.

Even if such a transfer were to take place, transfer of *bar*, *cryIA(c)*, *pinII*, or *bla* to a microorganism would pose no plant pest risk. The *bar*, *cryIA(c)*, *pinII*, and *bla* genes are all derived from microbes or plants found in nature.

F. Insect Resistance Management

DEKALB is aware of the possibility of the development of insect resistance to transgenic plants containing *B.t.* proteins. DEKALB is committed to working to continue the development and implementation of a plan to delay or eliminate insect resistance to *B.t.* corn. The U.S. Environmental Protection Agency's Office of Pesticide Programs established the Pesticide Resistance Management Workgroup (PRMW) in August of 1992 to review plans for insect resistance management submitted by registrants in support of new conventional, biological, or genetically engineered plant pesticides (Matten and Lewis, 1995). The PRMW identified seven elements which compose an effective insect resistance management (IRM) plan. These components were subsequently approved on March 1, 1995 by a subpanel of the FIFRA Scientific Advisory Panel (Matten and Lewis 1995). These elements include:

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

DEKALB has, and continues to develop strategies to prolong the utility of transgenic *B.t.* corn as a mechanism for control of lepidopteran pests. DEKALB believes that these strategies are consistent with the concerns about the development of insect resistance. DEKALB has submitted an extensive insect resistance management plan to the U.S. EPA. DEKALB will implement an IRM plan prior to commercial release of insect protected maize line DBT418.

IX. Statement of Grounds Unfavorable

DEKALB is unaware of any unfavorable grounds associated with insect protected corn line DBT418, developed using the plasmid vectors pDPG699, pDPG165 and pDPG320. Therefore, based on the potential benefits to the grower, the environment, and the consumer, DEKALB Genetics Corporation requests that the DBT418 corn line no longer be regulated under 7 CFR part 340.6.

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DEKALB Genetics Corporation

Petition for Determination of Nonregulated Status:

Insect Protected Corn *(Zea mays* L.) with the *cryIA(c)* Gene from *Bacillus thuringiensis* subsp. *kurstaki*

DEKALB #: DGC 96-059P

Appendices I, II, III and IV

Appendix I. DNA and Corresponding Amino Acid Sequence of the *cryIA(c)* Gene

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ENVIRONMENTAL RELEASE REPORT

Permit Number: 93-127-03N

Effective Date: 6/7/93

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: B1, BC, BD, BF, BI, BJ, BP.

Sites (with maximum acreage) and Periods of Release:

1) DeKalb County, IL (1 acre)
   6/93 - 11/93
2) Maui County, HI (1 acre)
   6/93 - 9/93
   10/93 - 2/94
   2/94 - 5/94

Purposes of Release:

a) Incorporate the transgenic locus into DEKALB proprietary germplasm.
b) Evaluate insect resistance of the transgenic lines.

Results:

a) The transgenic locus was back-crossed into DEKALB proprietary germplasm.
b) The degree of insect resistance of the transgenic lines were evaluated.

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or
Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers: All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-081-03N

Effective Date: 4/28/94

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release:

Maui County, HI (1 acre)
6/94 - 9/94
10/94 - 2/95

Purposes of Release:

a) To incorporate the transgenic locus into DEKALB proprietary germplasm.
b) To observe gene expression levels for the transgenes.

Results:

a) The transgenic locus was back-crossed into DEKALB proprietary germplasm.
b) Gene expression levels for the transgenes were evaluated.

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or
Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers: All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in Hawaii; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-081-06N

Effective Date: 4/28/94

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release:

1) New London County, CT (1 acre)
   6/2/94 - 10/1/94
2) DeKalb County, IL (1 acre)
   5/94 - 10/94
3) Champaign County, IL (0.8 acre)
   5/94 - 11/94
4) Adams County, NE (0.6 acre)
   5/6/94 - 10/12/94
5) Obion County, TN (1.2 acre)
   5/94 - 10/94

Purposes of Release:

a) incorporate the transgenic locus into DEKALB proprietary germplasm;
b) evaluate insect resistance of the transgenic line;
c) evaluate agronomic traits of the transgenic line (besides insect resistance);
d) observe gene expression levels for the transgenes;
e) determine segregation ratios of the transgenic locus;
f) set up demonstration plots.

Results:

a) the transgenic locus was put into DEKALB proprietary germplasm;
b) the degree of insect resistance of the transgenic line was evaluated;
c) agronomic traits of the transgenic line were evaluated;
d) gene expression levels for the transgenes were evaluated;
e) Mendelian segregation of the transgenic locus was observed;
f) demonstration plots of the transgenic lines were shown to the public.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or
Transgenic plants were either detassled or tassels were bagged.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed either by incorporation into the soil (e.g., repeated discing) or by burning.

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-115-02N

Effective Date: 5/25/94

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release:

1) McLean County, IL (0.5 acre)
   6/94 - 10/94
2) Lee County, IL (0.5 acre)
   6/94 - 10/94

Purpose of Release:

a) incorporate the transgenic locus into commercial germplasm;
b) evaluate insect resistance of the transgenic line;

Results:

a) the transgenic locus was put into commercial germplasm;
b) the degree of insect resistance of the transgenic line was evaluated;

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers: All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-220-02N

Effective Date: 9/21/94

Permittee:
DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BJ

Sites (with maximum acreage) and Periods of Release:

1) Palm Beach County, FL (1 acre)
   10/94 - 3/95
2) Maui County, HI (1 acre)
   10/94 - 2/95
   2/95 - 5/95
3) McLean County, IL (1 acre)
   5/95 - 9/95

Purpose of Release:

a) incorporate the transgenic locus into commercial germplasm;
b) evaluate insect resistance of the transgenic line.

Results:

a) the transgenic locus was put into commercial germplasm;
b) the degree of insect resistance of the transgenic line was evaluated.

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	The BJ18 line is resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or
Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers: All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America or Hawaii; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 94-243-01N

Effective Date: 9/30/94

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release: Dade County, FL (1 acre)
10/94 - 3/95

Purpose of Release: To evaluate insect resistance of the transgenic lines.

Results: The degree of insect resistance for each transgenic line was evaluated.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI and BJ lines are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species. microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-010-02N

Effective Date: 2/9/95

Permittee:
DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release:

1) Wayne County, NC (0.2 acre)
   4/11/95 - 10/95
2) Beaufort County, NC (0.2 acre)
   Not planted.

Purpose of Release:

The purposes of the release were the following:

a) evaluate insect resistance of the transgenic line;
b) evaluate agronomic traits of the transgenic line (besides insect resistance);
c) set up demonstration plots.

Results:

The results of the release were the following:

a) the degree of insect resistance of the transgenic line was evaluated;
b) agronomic traits of the transgenic line were evaluated;
c) demonstration plots of the transgenic lines were shown to the public.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	some plants showed susceptibility to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	herbicides were used
Adequate isolation:	was maintained
Water management:	irrigation (once)

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

Transgenic plants were either detassled or tassels were bagged.

Means of Plant Disposition:

Plant material was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-020-04N

Effective Date: 2/19/95

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI, BJ

Sites (with maximum acreage) and Periods of Release: Maui County, HI (1 acre)
3/95 - 6/95

Purpose of Release: Incorporate the transgenic locus into DEKALB proprietary germplasm.

Results: The transgenic locus was back-crossed into DEKALB proprietary germplasm.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal

Insect resistance: These lines are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in Hawaii; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-072-25N

Effective Date: 4/26/95

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI36, BJ18

Sites (with maximum acreage) and Periods of Release:

1) New London County, CT (1 site, 0.02 total acres)
   6/23/95 - 10/10/95
2) Lee County, GA (1 site, 0.01 total acres)
   4/27/95 - 9/95
3) DeKalb County, IL (2 sites, 1.05 total acres)
   5/5/95 - 10/20/95
   5/15/95 - 10/14/95
   5/31/95 - 10/13/95
4) Vermilion County, IL (1 site, 0.9 total acres)
   6/5/95 - 10/17/95
5) Sangamon County, IL (1 site, 0.9 total acres)
   Not planted
6) Mason County, IL (2 sites, 0.15 total acres)
   5/16/95 - 10/16/95
   6/8/95 - 10/16/95
7) Champaign County, IL (2 sites, 2.03 total acres)
   5/22/95 - 8/20/95
   5/22/95 - 10/16/95
   5/22/95 - 10/17/95
   6/95 - 11/10/95
8) Daviess County, IN (2 sites, 0.15 total acres)
   6/8/95 - 10/21/95
   6/16/95 - 10/19/95

9) Tipton County, IN (2 sites, 0.15 total acres)
   5/2/95 - 10/7/95
   4/29/95 - 10/18/95
10) Cass County, IA (2 sites, 0.15 total acres)
   5/15/95 - 10/11/95
   5/20/95 - 10/11/95
11) Webster County, IA (2 sites, 0.11 total acres)
   5/6/95 - 10/5/95
   5/16/95 - 10/19/95
   8/3/95 - 10/5/95
12) Johnson County, IA (2 sites, 0.15 total acres)
   5/16/95 - 10/2/95
   5/26/95 - 10/24/95
13) Clay County, IA (1 site, 0.01 total acres)
   Not planted
14) Haskell County, KS (1 site, 0.01 total acres)
   5/23/95 - 6/95
   Plants destroyed at emergence.
15) Ingham County, MI (1 site, 0.01 total acres)
   5/14/95 - 10/5/95
16) Clay County, MN (1 site, 0.01 total acres)
   Not planted
17) Renville County, MN (1 site, 0.01 total acres)
   5/12/95 - 9/28/95
   5/26/95 - 11/2/95
18) Steele County, MN (1 site, 0.01 total acres)
   8/4/95 - 10/20/95
19) Saline County, MO (1 site, 0.01 total acres)
   6/1/95 - 9/15/95
20) Adams County, NE (2 sites, 0.15 total acres)
   5/19/95 - 10/17/95
   5/20/95 - 11/14/95
21) Marion County, OH (1 site, 0.01 total acres)
   Not Planted.
22) Lancaster County, PA (1 site, 0.01 total acres)
   5/9/95 - 10/20/95
23) Minnehaha County, SD (1 site, 0.01 total acres)
   5/19/95 - 10/25/95
24) Obion County, TN (2 sites, 0.15 total acres)
   5/11/95 - 10/95
   5/17/95 - 10/95
   6/13/95 - 10/95
25) Weakley County, TN (2 sites, 0.15 total acres)
   5/12/95 - 10/95

26) Columbia County, WI (1 site, 0.01 total acres)
5/22/95 - 10/15/95

Purpose of Release:

The purposes of the release were the following:

a) incorporate the transgenic locus into DEKALB proprietary germplasm;
b) evaluate insect resistance of the transgenic line;
c) evaluate agronomic traits of the transgenic line (besides insect resistance);
d) observe gene expression levels for the transgenes;
e) determine segregation ratios of the transgenic locus;
f) set up demonstration plots.

Results:

The results of the release were the following:

a) the transgenic locus was put into DEKALB proprietary germplasm;
b) the degree of insect resistance of the transgenic line was evaluated;
c) agronomic traits of the transgenic line were evaluated;
d) gene expression levels for the transgenes were evaluated;
e) Mendelian segregation of the transgenic locus was observed;
f) demonstration plots of the transgenic lines were shown to the public;

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	now resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides were applied at some sites
Adequate isolation:	was maintained
Water management:	irrigation at some sites

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

At the different locations, one (or more) of the following methods were employed:

a) *Spacial:* In agronomic performance tests, transgenic corn was open-pollinated and spatially isolated by at least 660 feet from corn that was not involved in the performance test.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes; seed and silage for nutritional analysis); or

b) was destroyed by either incorporation into the soil (e.g., repeated discing) or burning.

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-072-26N

Effective Date: 4/17/95

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI36, BJ18, B16, A18, A24.

Sites (with maximum acreage) and Periods of Release:

1) New London County, CT (1 site, 0.12 total acres)
   6/23/95 - 10/10/95
2) Tazewell County, IL (1 site, 0.45 total acres)
   5/2/95 - 10/2/95
3) DeKalb County, IL (1 site, 0.05 total acres)
   5/22/95 - 10/30/95
4) Renville County, MN (1 site, 0.11 total acres)
   5/3/95 - 10/10/95
   5/16/95 - 10/10/95
5) Obion County, TN (1 site, 0.15 total acres)
   4/28/95 - 10/95

Purpose of Release:

The purposes of the release were the following:
- a) evaluate insect resistance of the transgenic line;
- b) evaluate agronomic traits of the transgenic line (besides insect resistance);
- c) observe gene expression levels for the transgenes;
- d) determine segregation ratios of the transgenic locus;
- e) set up demonstration plots.

Results:

The results of the release were the following:

a) the degree of insect resistance of the transgenic line was evaluated;
b) agronomic traits of the transgenic line were evaluated;
c) gene expression levels for the transgenes were evaluated;
d) Mendelian segregation of the transgenic locus was observed;
e) demonstration plots of the transgenic lines were shown to the public;

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides were applied at some sites
Adequate isolation:	was maintained
Water management:	irrigation at some sites

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

At the different locations, one (or more) of the following methods were employed:

a) *Spacial:* In agronomic performance tests, transgenic corn was open-pollinated and spatially isolated by at least 660 feet from corn that was not involved in the performance test.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes; seed and silage for nutritional analysis); or

b) was destroyed by either incorporation into the soil (e.g., repeated discing) or burning.

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-111-07N

Effective Date: 5/26/95

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI36, BJ18

Sites (with maximum acreage) and Periods of Release: Dixon County, NE
(1 site, 0.5 total acres)
6/95 - 10/95

Purpose of Release:

Determine the effect of Bt-containing corn on non-target insects.

Results:

The populations of non-target insects were measured on transgenic and nontransgenic corn; no significant differences were observed.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change

Ability to outcross:	no change
Survival of progeny in the field:	normal
Insect resistance:	BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations where transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

Means of Plant Disposition:

Plant material was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in North America; therefore, transfer to wild weedy relatives is not possible.

ENVIRONMENTAL RELEASE REPORT

Permit Number: 95-138-02N

Effective Date: 6/17/95

Permittee: DEKALB Plant Genetics
3100 Sycamore Road
DeKalb, IL 60115
(815) 758-7333

Transgenic Lines: BI36, BJ18

Sites (with maximum acreage) and Periods of Release: Maui County, HI
(1 site, 6 total acres)
7/95 - 10/95

Purpose of Release: Incorporate the transgenic locus into DEKALB proprietary germplasm.

Results: The transgenic locus was put into DEKALB proprietary germplasm.

Observations:

Plant Characters Monitored for Changes:

Reproductive traits:	no change
Disease status:	no change
Morphological traits:	no change
Herbicide resistance:	now resistant to glufosinate
Weediness:	no change
Ability to outcross:	no change
Survival of progeny in the field:	normal

Insect resistance: BI36 and BJ18 are resistant to feeding by larvae of European corn borer (*Ostrinia nubilalis*)

Any evidence that inserted DNA has been transferred to other species, microbial or plant? No

Monitoring of Field Containment:

Wildlife or livestock invading field:	none
Insect or weed control:	pesticides and herbicides
Adequate isolation:	was maintained
Water management:	irrigation

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

One (or more) of the following methods were employed:

a) *Spacial:* Transgenic corn was open-pollinated and spatially isolated by at least 660 feet from other corn.

b) *Physical:* In situations were transgenic plants were shedding pollen, ear shoots of all corn plants within 660 feet of were ear-bagged; and/or Transgenic plants were either detassled or tassels were bagged.

c) *Temporal:* Transgenic plants were planted at a significantly different time from adjacent nontransgenic plants, to prevent cross-pollination.

Means of Plant Disposition:

Plant material was either:

a) stored for future use (e.g., seed for breeding purposes); or

b) was destroyed by incorporation into the soil (e.g., repeated discing).

Monitoring for Volunteers:

All volunteer plants were destroyed prior to anthesis.

Additional Comments:

No other plant species that can cross-pollinate with corn are present in Hawaii; therefore, transfer to wild weedy relatives is not possible.

Appendix III, Table 1. Diseases Detected at DBT418 Field Release Locations During 1993 [a,b]

DBT418 Release Location		Foliar Diseases														Ear Rot								Stalk Rot						
County	State	Helminthosporium Leaf Spot	Common Corn Rust	Stewart's Disease	Common Corn Smut	Anthracnose	Southern Corn Leaf Blight	Northern Corn Leaf Blight	Helminthosporium Leaf Disease	Eyespot	Southern Corn Rust	Zonate Leaf Spot	Goss's Wilt	Holcus Spot	Gray Leaf Spot	Fusarium Ear Rot	Gibberella Ear Rot	Penicillium Ear Rot	Trichoderma Ear Rot	H. carbonum Ear Rot	Cladosporium Ear Rot	Nigrospora Ear Rot	Diplodia Ear Rot	Gibberella Stalk Rot	Fusarium Stalk Rot	Charcoal Stalk Rot	Anthracnose Stalk Rot	Nigrospora Stalk Rot	H. carbonum Stalk Rot	Red Root Rot
DeKalb	IL	14	1234		X	1234	234	34		134		34			4	X	X				X			X	X		X	X		X

(a) Sampling times at which a particular disease was detected: 1 = knee-high; 2 = mid-silk; 3 = 2 weeks after mid-silk; 4 = 4 weeks after mid-silk; X = harvest
(b) Information is not available for Maui County, HI.

Appendix III, Table 2. Diseases Detected at DBT418 Field Release Locations During 1994 [a,b]

DBT418 Release Location		Foliar Diseases														Ear Rot								Stalk Rot						
County	State	Helminthosporium Leaf Spot	Common Corn Rust	Stewart's Disease	Common Corn Smut	Anthracnose	Southern Corn Leaf Blight	Northern Corn Leaf Blight	Helminthosporium Leaf Disease	Eyespot	Southern Corn Rust	Zonate Leaf Spot	Goss's Wilt	Holcus Spot	Gray Leaf Spot	Fusarium Ear Rot	Gibberella Ear Rot	Penicillium Ear Rot	Trichoderma Ear Rot	H. carbonum Ear Rot	Cladosporium Ear Rot	Nigrospora Ear Rot	Diplodia Ear Rot	Gibberella Stalk Rot	Fusarium Stalk Rot	Charcoal Stalk Rot	Anthracnose Stalk Rot	Nigrospora Stalk Rot	H. carbonum Stalk Rot	Red Root Rot
Champaign	IL	3	234	234	13		23	14							234	X								X	X		X			X
DeKalb	IL	1	1234		2		123	234		234					4	X								X	X		X			X
Adams	NE	2	1234			4				4					4		X			X							X			X
Obion	TN	23	23	123	1		1								3	X							X			X			X	

(a) Sampling times at which a particular disease was detected: 1 = knee-high; 2 = mid-silk; 3 = 2 weeks after mid-silk; 4 = 4 weeks after mid-silk; X = harvest
(b) Information is not available for New London County, CT, McLean County, IL, Lee County, IL, Maui County, HI, Palm Beach County, FL, and Dade County, FL.

Appendix III, Table 3. Diseases Detected at DBT418 Field Release Locations During 1995 [a,b]

DBT418 Release Location		Foliar Diseases														Ear Rot								Stalk Rot						
County	State	Helminthosponum Leaf Spot	Common Corn Rust	Stewart's Disease	Common Corn Smut	Anthracnose	Southern Corn Leaf Blight	Northern Corn Leaf Blight	Helminthosporium Leaf Disease	Eyespot	Southern Corn Rust	Zonate Leaf Spot	Goss's Wilt	Holcus Spot	Gray Leaf Spot	Fusarium Ear Rot	Gibberella Ear Rot	Penicillium Ear Rot	Trichoderma Ear Rot	H. carbonum Ear Rot	Cladosporium Ear Rot	Nigrospora Ear Rot	Diplodia Ear Rot	Gibberella Stalk Rot	Fusarium Stalk Rot	Charcoal Stalk Rot	Anthracnose Stalk Rot	Nigrospora Stalk Rot	H. carbonum Stalk Rot	Red Root Rot
Lee	GA	124	234			1234	34	124			4					X									X		X			
Cass	IA	134	34	234		14	1			234					234	X														X
Clay	IA	1	4			14	4	34	34						4	X														
Johnson	IA	3	34			1	234	234		3					234												X			X
Webster	IA	234	234			23	234	34		234	4				34	X											X			X
Champaign	IL	1	2	1234	1	1									234	X							X	X		X				X
DeKalb	IL	24	23	3	2	24	234	34		2					234	X								X	X		X			X
Haskell	KS	24	24			2																								
Ingham	MI	14		234		124		34		1234					234															X
Renville	MN		4		123	1		234		234						X	X							X				X		X
Stelle	MN	23	4		3	2		234		234					4	X								X			X			X
Saline	MO	2	23	23		3									3															
Wayne	NC	12	23	3	12	3	23	3	2																					
Adams	NE	4	234	234	3										34	X								X			X			X
Lancaster	PA	234	234	4	3	234	4								3	X									X					X
Minnehaha	SD		234		34					234					4	X		X						X						X
Obion	TN	13	134	34			3	3							34								X	X						

(a) Sampling times at which a particular disease was detected: 1 = knee-high; 2 = mid-silk; 3 = 2 weeks after mid-silk; 4 = 4 weeks after mid-silk; X = harvest

(b) Information is not available for Dade County, FL, Maui County, HI, New London County, CT, Tazewell County, IL, Dixon County, NE, Vermilion County, IL, Mason County, IL, Daviess County, IN, Tipton County, IN, Weakley County, TN, and Columbia County, WI.

Appendix III, Table 4. Insect Pests Present at DBT418 Field Release Locations - 1995 [a,b]

DBT418 Release Location		Insect Pest																
County[b]	State	Sugar cane borer	Corn rootworm	European corn borer	Lesser stalk corn borer	Southwestern corn borer	Corn earworm	Fall armyworm	Black cutworm	Aphid	Seedcorn maggot	Wireworm	Flea beetle	White grub	Japanese beetle	Spidermite	Stinkbug	Grasshopper
New London	CT									1								
Dade	FL	1					3	2		X						X		
Lee	GA						1			X							X	
Kauai	HI	X			X		1	2		3						4	X	
Maui	HI						2			1					3	4		
Cass	IA			X														
Johnson	IA			1						X							X	
Webster	IA		X	1					X	X			X					
Champaign	IL		X										X					
DeKalb	IL		X	1			X		X	X	X		X	X				
Mason	IL		X	2						X			1		X		X	
Tazewell	IL		X	2						X			1		X		X	
Vermilion	IL		X										X		X			
Daviess	IN		X	X						X			X		X			
Tipton	IN		3	1			X		X	X			2					
Ingham	MI		X	X														
Renville	MN			1						X								2
Steele	MN		X	X						X								
Wayne	NC		1				2	X	X	X	X	X	3	X	X	X	X	
Adams	NE		X	1						X						X		
Lancaster	PA		2	1					3									
Minnehaha	SD		2	1			4			3								
Obion	TN			2		1	3	4		X			5				X	
Columbia	WI		X	X														

(a) Numbered in order of importance (i.e., "1" denotes most severe insect pest). "X" denotes the insect is present but causes less than 5% of insect damage to crop.
(b) Information is not available for Saline County, MO.

Appendix IV. Effect of Corn Line DBT418 on Lepidopteran Pests

As described in the petition, insect protected corn line DBT418 offers good control of European corn borer (ECB). Little or no leaf feeding or stalk tunneling is observed as the result of first or second generation ECB infestations of DBT418 plants. DBT418 corn also provides significant control of Southwestern corn borer (SWCB). First generation SWCB leaf feeding is greatly reduced and tunneling is reduced in second generation infestations. Growth inhibition of corn earworm (CEW) has been observed as the result of silk and ear feeding on DBT418 plants. DBT418 does not control fall armyworm (FAW). Field and bioassay data describing the level of control of each of these insect pests by insect protected corn line DBT418 are presented below.

European Corn Borer

DEKALB evaluated the control of first and second generation ECB on insect protected corn line DBT418 in 1994 (Table 1). The experiment was performed at two locations, Thomasboro, IL and Glenvil, NE. Data presented for the first generation ECB ratings are from Thomasboro only (no first generation evaluation was performed at Glenvil). This experiment included a number of factors (including other transformed lines), but only those factors relevant to this discussion are included here. The test was arranged as a split-split plot (main factor being infestation level) with three replications at each location. Subplots consisted of transformant sources (only data on infestation of the DBT418 event is presented). Sub-subplots consisted of paired 2-row comparisons of identical genotypic lines with and without the DBT418 insertion event. Rows consisted of 30 plants. The DBT418 genotype tested was a partially converted hybrid containing the DBT418 insertion event. The control genotype was a comparable partially converted hybrid lacking the transgene insertion.

For the first generation ECB simulation, plants were inoculated three times at mid-whorl stage. Fifty neonate larvae dispersed in corn cob grits were introduced to the whorl of the plant on three separate days for a total of 150 larvae. Leaf damage ratings for first generation inoculations were scored on all plants in both rows of each replication, one week after the last ECB infestation using the 1 to 9 Guthrie scale where: 1= no damage to fine shot hole scars, and 9 = elongated lesions (> 1") on most leaves. The DBT418 line provided good protection from first generation ECB damage (Table 1). For the second generation ECB simulation, neonate larvae were again mixed with corn cob grits and applied to leaf axils near the ear at anthesis. Plants were inoculated with fifty larvae on two separate days for a total of 100 larvae per plant. Stalk tunneling was measured just before harvest. Ten plants in each sub-subplot were scored for the number of tunnels and total centimeters of tunneling in the stalk from ground level to two nodes above the ear. The DBT418 line had significantly fewer tunnels, and significantly less tunnel damage than the nontransgenic control (Table 1).

Evaluation of DBT418 plants for ECB control was repeated in 1995 (Table 2). A large experiment was performed at two locations, Thomasboro, IL and Georgetown, IL. Again, this experiment included a number of factors (including other transformed lines), but only those factors relevant to this discussion are included here. The test was arranged as a split-split plot with four replications at each location. The factors examined in this experiment included corn borer treatments, either artificially infested with insects or uninfested (data on the uninfested plots is not shown), transformant sources (only data on the DBT418 event is presented) and comparisons of identical genotypic lines (planted as three row plots) with and without the DBT418 insertion event.

For the first generation ECB simulation, plants were inoculated three times at the mid-whorl stage. Fifty neonate larvae dispersed in corn cob grits were introduced to the whorl of the plant at each inoculation. Leaf damage ratings for first generation inoculations were scored one week after the last ECB infestation to each sub-plot using a modified 0 to 9 Guthrie scale. Because of the extreme level of protection conferred by transgenic insect protected lines, a modified Guthrie damage rating system was used in which a 0 rating represents no visible leaf feeding damage. For each replication, ratings were taken on all three rows (32 plants per row). As observed in the previous year, DBT418 provided good protection from first generation ECB infestation (Table 2). For the second generation ECB simulation, neonate larvae were again mixed with corn cob grits and applied to leaf axils near the ear at anthesis. Inoculations of 50 larvae per plant were performed on two different days (100 larvae total). Stalk tunneling was measured just before harvest. Ten plants per replication (taken from one of the three rows) of each genotype were scored for the number of tunnels and total centimeters of tunneling in the stalk from ground level to two nodes above the ear. As observed in the 1994 test, the DBT418 line had significantly fewer tunnels, and significantly less tunnel damage than the nontransgenic control (Table 2).

An experiment was performed by DEKALB in 1996 to compare the level of protection conferred by a DEKALB hybrid containing the DBT418 insertion event, a commercially available Bt hybrid (Ciba Seeds hybrid Maximizer 454), and a non-transgenic control hybrid. Hybrids were inoculated with a simulated second generation ECB infestation at five separate timepoints similar to the range the might be encountered in the field under various circumstances (Table 3). Plants were inoculated at the following five growth stages:

Mid Silk Stage
Blister Stage (10 days post mid silk)
Milk Stage (20 days post mid silk)
Dough Stage (30 days post mid silk)
Dent Stage (40 days post mid silk)

The experiment was planted in three replications at Union City, Tennessee in a split plot design with whole plots as the stage of infestation and subplots as the different genotypes.

Each subplot consisted of a three row planting of each genotype but only the center row was infested and evaluated for ECB tunneling damage. One hundred fifty to two hundred neonate ECB larvae, dispersed in corn cob grits were inoculated to leaves close to the ear of each plant. Tunneling was determined on stalks split at harvest on ten plants per subplot (30 plants of each genotype were sampled for each inoculation stage to determine tunneling).

The stalk tunneling means in Table 3 demonstrate the level of protection conferred by a hybrid containing the DBT418 event compared to the commercially obtained Maximizer 454 hybrid and a non-transgenic control hybrid at various stages in late-season development. The two transgenic lines were approximately equivalent in their level of protection. Both lines demonstrated statistically significant protection from stalk tunneling at the Blister, Dent, and MidSilk stages of plant development. Neither line was statistically better than the control line at Dough stage (although mean tunneling in both transgenic lines was less that on half the control) while DBT418 exhibited less tunneling than Maximizer 454 or the control hybrid at Milk stage. These results illustrate the comparable efficacy of DBT418 and a commercial Bt transgenic line in providing protection from second generation ECB damage.

In an experiment performed at DEKALB's Olivia, MN station in 1996, larval survival on a DEKALB hybrid containing the DBT418 insertion event was compared to larval survival on Maximizer 454 (Table 4). Plants were heavily artificially infested to simulate second generation ECB infestation and then sampled two and a half weeks later to determine the number of surviving larvae. Plants were inoculated on three separate days with 40 larvae (120 total). For evaluation, five plants in each row were completely stripped of leaves and the ears and stalks dissected to look for larvae. The experiment was set up as two paired rows of each genotype per replicate with three replicates for a total of six rows and 30 plants of each genotype dissected. This data demonstrated that both lines provided statistically significant reduction in the number of larvae present in vegetative tissues. However, the data also indicates that larvae can survive in the ear of both transgenic lines.

The advantage of transgenic Bt corn over conventional pesticides has been well documented (Higgins et al., 1995; Higgins et al., 1994; Lotstein, 1994). Experiments were performed by DEKALB in 1996 to compare the level of first and second generation ECB control provided by DBT418 versus conventional insecticide application (Table 5). The experiments are not yet complete but limited data is available, however statistical analysis has not been performed. A comparison of control of first generation ECB infestation was performed at Union City, TN. The test was designed as a randomized complete block with three replications. Hybrids were planted in four row plots, 20 feet in length. Insecticide application and data collection was performed using the center two rows in each plot. Plots were 20 feet long and thinned to 28 plants per row. Approximately one month after planting each plant was infested with approximately 200 neonate ECB larvae. Two days after infestation, insecticides (Dipel 10G and Pounce 1.5G) were applied to the appropriate rows. Leaf feeding ratings, using the modified 0 to

9 Guthrie scale, were taken approximately two weeks after infestation. Leaf feeding was reduced in the insecticide treatments as compared to the nontransgenic control (Table 5). The leaf feeding score for the DBT418 treatment was very low, less than the pesticide treatments and the nontransgenic control (Table 5).

The second generation infestation experiment to compare DBT418 to insecticides was carried out at three locations: Union City, TN, Clay Center, NE, and Meade, NE. The Union City location was handled by DEKALB and the tests in Nebraska were conducted by Dr. John Foster, University of Nebraska. The experiment was planted as a three replication, randomized complete block design. Hybrids were planted in four row plots, 20 feet in length thinned to 28 plants. Pesticide application and data collection was performed using the center two rows in each plot. No insecticide application was necessary to control natural first brood infestation as monitored by slight first generation moth flight. Insecticide applications, Pounce 1.5G and Dipel 1.5G, were performed at the time of second brood moth flight. The second generation data represents 80 plants per treatment. Three replications of ten plants each were performed for each treatment at each location except Clay Center. At Clay center, one replication was lost to early season flooding. The data has not yet been statistically analyzed but indicates that the control provided by DBT418 greatly exceeds that provided by pesticides (Table 5). The pesticide treatments in the second generation experiment do not appear to differ significantly from the control.

Table 1. Evaluation of first and second generation ECB damage to DBT418 and control hybrid plants following artificial infestation in 1994[1].

Line	First Generation Rating	Second Generation	
		No. Tunnels/Plant	Cm. Tunneling/Plant
Control	5.3a	3.7a	10.1a
DBT418	1.0b	0.8b	1.8b

[1] Means under a single heading followed by different letters differ at P=0.01. Information in this table was extracted from a more extensive ANOVA analysis involving several transformant sources.

Table 2. Evaluation of first and second generation ECB damage following artificial infestation in 1995[1].

Line	First Generation Rating	Second Generation	
		No. Tunnels/Plant	Cm. Tunneling/Plant
Control	6.6a	2.3a	10.5a
DBT418	0.9b	0.5b	1.7b

[1] Means under a single heading followed by different letters differ at P=0.01. Information in this table was extracted from a more extensive ANOVA analysis involving several transformant sources.

Table 3. Comparison of second generation ECB protection by a DBT418 Hybrid, Maximizer 454, and a Nontransgenic Control Hybrid following infestation at various timepoints.

Genotypes	Plant Growth Stages Evaluated[1,2]				
	MidSilk	Blister	Milk	Dough	Dent
DBT418	5.1a	0.8a	3.1a	1.7a	0.7a
Maximizer 454	2.0a	3.6a	10.1b	2.1a	0.6a
Control	13.3b	9.5b	9.8b	4.4a	7.2b

[1] Mean stalk tunnel lengths in cm.
[2] Means in a column followed by the same letter are not significantly different at P= .001 as determined by Tukey's HSD procedure.

Table 4. Comparison of ECB Larval Survival on a DBT418 Hybrid, Maximizer 454, and a Nontransgenic Control Hybrid[1].

Corn Hybrid	Number of Surviving ECB Larvae	
	From Vegetative Tissues	From Ear Tissue
Control Hybrid	1.8a	9.4b
DBT418 Hybrid	0.13b	5.8b
Maximizer 454	0.03b	4.9b

[1] Means under a single heading followed by different letters differ at P=0.01 using Tukey's HSD procedure.

Table 5. A Comparison of Control of First and Second Generation ECB by DBT418 and Conventional Insecticides

Treatment	First Generation Infestation: Leaf Damage Rating	Second Generation Infestation: Ave. Cm. Tunneling per Plant
Control Hybrid	4.6	11.7
Control Hybrid - Dipel	2.0	13.9
Control Hybrid - Pounce	2.3	11.1
DBT418 Hybrid	0.2	2.8

Southwestern Corn Borer

Results from field studies performed by DEKALB in 1994 demonstrated significant reduction in feeding and plant damage on DBT418 plants resulting from first and second generation SWCB infestations (Tables 6 and 7). These tests were performed at the DEKALB breeding station in Union City, TN. DBT418 plants used in the test were incomplete conversions of an elite inbred. Nontransgenic plants of the same inbred genotype were used as the control. Plants were infested with 30 neonate SWCB larvae per plant. The data was collected from a split plot design with 10 ft. rows, four replications per treatment, approximately ten plants per replication. First generation evaluations were performed by infesting whorl-stage plants and evaluating feeding damage and larval weights eight days after infestation. In a separate test, second generation evaluations were performed by infesting plants at flowering and evaluating feeding damage 28 days after infestation. Results from first generation SWCB infestations demonstrated greatly reduced leaf feeding and tunneling in DBT418 plants, compared to nontransgenic controls (Table 6). The average number of larvae and average larval weight was also greatly reduced on the transgenic plants as compared to the controls. In the second generation SWCB infestation, tunneling was significantly reduced on DBT418 plants compared to nontransgenic controls (Table 7).

Southwestern corn borer studies were repeated by DEKALB at the Union City location in 1995 (Tables 8 and 9). Results from first and second generation SWCB infestations on DBT418 plants were similar to results obtained in 1994. A DEKALB hybrid containing the DBT418 insertion event was compared to control plants of the same hybrid lacking the insertion event. First and second generation infestations were performed in separate tests by inoculating transgenic and control plants with 30 neonate SWCB larvae. In the first generation infestation experiment, leaf feeding, number of larvae, and average larval weight was determined 14 days after inoculation (Table 8). Leaf feeding, number of larvae, and larval weight were greatly reduced on DBT418 plants compared to the control. In the second generation infestation experiment, 25 plants each of the DBT418 and control hybrids were analyzed for tunneling damage (Table 9). As observed in the 1994 tests, the relative damage as the result of second generation SWCB infestations was greater than that observed in first generation infestations, however, significant protection was provided.

Table 6. Results from first generation SWCB infestation of DBT418 and control plants performed at Union City in 1994[1].

Line	Leaf Feeding[2]	Ave Larval Weight (mg)	Ave. No. Larvae/Plant	Ave. Total Larval Weight/Plant (mg)	Ave. No. Internodes Tunneled/ Plant
Control	9a	11.9a	36.3a	423.7a	3.3
DBT418	2b	0.8b	2.5b	2.2b	0.3

[1] Means under a single heading followed by different letters differ at P=0.01 by LSD.
[2] scale of one to nine, nine being most severe damage

Table 7. Result from second generation SWCB infestation of DBT418 and control plants performed at Union City in 1994[1].

Line	Tunnels/Plant (cm)	% of Plants with Husk Feeding
Control	14.4a	95a
DBT418	5.2b	78a

[1] Means under a single heading followed by different letters differ at P=0.01 by LSD.

Table 8. Results from first generation SWCB infestation of DBT418 and control plants performed at Union City in 1995[1].

Line	Leaf Feeding[2]	Ave Larval Weight (mg)	Ave. No. Larvae/Plant	Ave. Total Larval Weight/Plant (mg)
Control Unconverted Hybrid	9.0a	33.8a	5.3	179.1
DBT418 Hybrid	1.7a	2.1a	0.4	0.8

[1] means under a single heading followed by different letters differ at P=0.01 using LSD.
[2] scale of one to nine, nine being most severe damage

Table 9. Results from second generation SWCB infestation of a DBT418 hybrid and a control hybrid performed at Union City in 1995.

Line	% Plants Tunneled	Ave. Cm. Tunneling/Plant	Ave. Cm. Tunneling/Infested Plant
Unconverted Hybrid	100	26.7	26.7
DBT418 Hybrid	44	4.7	10.6

Corn Earworm

A field study performed at the DEKALB breeding station in Union City, TN in 1995 indicated a trend towards reduction in CEW larval weight gain on DBT418 plants, as compared to nontransgenic control plants, following infestation of ears on green silk stage plants with neonate CEW larvae (Table 10). Inbred plants converted with the DBT418 insertion event were compared to the nontransgenic version of the same inbred; seven pairs were compared. Plants were infested with five CEW larvae by placing the larvae directly on the silks. Data was collected ten days after infestation. The establishment of CEW on transgenic and nontransgenic plants was roughly equivalent. A general reduction in larval weight was observed on DBT418 plants as compared to control plants, however the difference between the overall means was not statistically significant. CEW larvae feeding on DBT418 silk and ears exhibited an average larval weight that was approximately 40% less than that of the controls.

The effect of DBT418 plants on CEW was further investigated in 1996 in laboratory and field tests performed for DEKALB by Dr. Bob Lynch and Dr. Billy Wiseman, USDA-ARS (Insect Biology and Population Management Research Lab, Coastal Plain Experiment Station, Tifton, GA). The survival and weight of CEW larvae on hybrid plants containing the DBT418 insertion event was evaluated in the laboratory using excised leaf tissue (Table 11) and silks (Table 12). A nontransgenic version of the same hybrid was used as the control. In each experiment, larvae were cultured individually, one larvae per cup of diet. A total of 30 larvae were tested for each treatment (five replications of six larvae each). Significant reduction in CEW larval weight was observed for DBT418 leaf tissue compared to the control (Table 11). Treatments included neonate larvae feeding on fresh leaf tissue, neonate larvae feeding on oven-dried leaf tissue incorporated into diet, 3-day old larvae feeding on oven-dried leaf tissue incorporated into diet, and 6-day old larvae feeding on oven-dried leaf tissue incorporated into diet. A similar experiment was conducted using silks excised from DBT418 and control plants (Table 12). The growth of neonate larvae was reduced on DBT418 compared to control plants following feeding on fresh silks or silks incorporated into diet. Three-day old CEW larvae were affected by DBT418 silk incorporation into diet but not by fresh DBT418 silks, and 6-day old larvae were unaffected by DBT418 silks, fresh or incorporated into diet. The effect of DBT418 plants on CEW was further investigated by evaluating the number of larvae per ear and extent of ear damage following infestation of a DBT418 hybrid and the nontransgenic version of the same hybrid with CEW eggs (Table 13). Plants were infested at the silk stage by placing approximately 35 CEW eggs directly on the silks. Plants were evaluated 18 days after infestation. No significant reduction in number of larvae per ear, number of ears damaged, or extent of damage was observed in the comparison of DBT418 to the control.

Table 10. CEW establishment and determination of larval weights follow infestation of DBT418 and control plants

Line	No. Plants Sampled	% Infested	Ave. Larval Weight (mg)
DJ	38	66	92.0
DJ-DBT418	7	71	43.4
AW	6	67	76.9
AW-DBT418	4	75	52.5
BK	6	50	153.1
BK-DBT418	3	100	66.6
DI	29	86	47.5
DI-DBT418	19	63	47.2
CD	10	80	62.6
CD-DBT418	14	64	40.3
BS	26	67	159.4
BS-DBT418	15	67	51.1
BT	46	59	148.6
BT-DBT418	10	50	159.5
AVERAGE:			
Control Inbreds		68*	105.7**
DBT418 Inbreds		70*	65.8**

* means were not significantly different using Tukey's multiple range test.
** means were not significantly different by LSD (0.05).

Table 11. Survival and weight of corn earworm larvae when reared on a diet containing leaves of DBT418 plants in the laboratory.[1]

Corn Line	Neonates					3-day-old larvae		6-day-old larvae
	Fresh leaves		12 g oven-dried leaves/ 500 ml diet	24 g oven-dried leaves/ 500 ml diet	30 g fresh leaves/ 500 ml diet	30 g fresh leaves/ 400 ml diet		30 g fresh leaves/ 400 ml diet
	No. alive at 9 days	Weight (mg) of larvae at 9 days				No. alive at 9 days	Weight (mg) of larvae at 9 days	Weight (mg) of larvae at 10 days
DBT418 Hybrid	0.97 a	40.3 a	11.0 a	5.2 a	135.3 a	1.0 a	86.3 a	433.1 a
Control Hybrid	1.00 a	688.1 b	225.0 b	315.5 b	539.9 b	1.0 a	528.2 b	715.6 b
Bean Diet Check	1.00 a	779.0 c	331.7 c	356.7 b	722.9 c	1.0 a	557.7 b	727.6 b

[1] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$) using Waller-Duncan k-ratio t test.

Table 12. Weight of corn earworm larvae and silk damage rating when fed silks or silk diets of DBT418 plants in the laboratory.[1]

Corn line	Neonates			3-day-old larvae		6-day-old larvae		
	Fresh silks	80 g fresh silks/400 ml diet	20 g oven-dried silks/400 ml diet	Fresh silks	80 g fresh silks/400 ml diet	Fresh silks		80 g fresh silks/400 ml diet
	Weight (mg) of larvae at 7-9 days			Weight (mg) of larvae at 10 days		Damage Rating[2]	Weight (mg) of larvae at 10 days	
DBT418 Hybrid	31.7 a	55.6 a	0.9 a	343.7 a	135.4 a	4.67 a	445.0 a	472.9 a
Control Hybrid	177.3 b	111.9 b	12.4 a	343.3 a	276.3 b	4.79 a	437.5 a	592.2 a
Bean Diet Check	-	729.8 c	772.6 b	-	836.8 c	-	-	542.0 a

[1] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$) using Waller-Duncan k-ratio t test.
[2] Damage rated on a 1-5 scale where 1 = no damage and 5 = extensive damage.

Table 13. Ear damage caused by the corn earworm on DBT418 plants.[1]

Corn line	Corn earworm		
	No. larvae/ear	No. ears damaged	Damage (cm)
DBT418 Hybrid	0.23 a	0.80 a	4.93 a
Control Hybrid	0.37 a	1.00 a	4.23 a

[1] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$) using Waller-Duncan k-ratio t test.

Fall Armyworm

DBT418 plants were evaluated for control of FAW in an experiment performed in 1996 by Dr. Robert Lynch and Dr. Billy Wiseman, USDA-ARS. FAW larvae were evaluated in the laboratory for their ability to survive on leaf tissue excised from transgenic and control plants (Table 14). Each treatment consisted of a total of 30 larvae (five replications of six larvae each). Neonate larvae were cultured two larvae per cup of diet; older larvae were cultured one larvae per cup. No difference in larval survival or weight, or larval damage to leaves was observed in the laboratory test. In the field portion of the study, DBT418 hybrid plants were infested on two consecutive days with 20 neonate FAW larvae per infestation and evaluated for FAW feeding damage 7 and 14 days following infestation (Table 14). No difference in FAW feeding on DBT418 versus the control was observed at either 7 or 14 days.

Table 14. Survival, weight, and damage rating in laboratory and field evaluations of fall armyworm larvae on leaves of DBT418 plants in the mid-whorl stage of development.[1]

Corn line	Neonates	3-day-old larvae	6-day-old larvae		Plant damage rating (Field)[3]	
	No. alive after 4 days on leaves	No. alive after 4 days on leaves	Weight (mg) of larvae at 10 days	Damage rating to leaves (Lab)[2]	7 days after inoculation	14 days after inoculation
DBT418 Hybrid	2.00 a	0.93 a	251.0 a	4.90 a	7.83 a	9.00 a
Control Hybrid	1.90 a	1.00 a	266.0 a	4.77 a	7.50 a	8.83 a

[1] Means within a column followed by the same letter are not significantly different ($P \leq 0.05$) using Waller-Duncan k-ratio t test.
[2] Damage rated on a 1-5 scale where 1 = no damage and 5 = extensive damage.
[3] Damage rated on a 1-9 scale where 1 = no damage and 9 = whorl and furl leaves almost totally destroyed.

References

Higgins, R., Buschman, L., Bowling, R., and Sloderbeck, P. 1995. Evaluation of Bt Corn in Western Kansas, 1995. Kansas State University, Southwest Research Extension Center Publication.

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