95-145-01

Petition for Determination of Nonregulated Status:

Glufosinate Resistant Corn Line B16

DG# 95-128P

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

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Contains No Confidential Business Information

Summary:

DEKALB Genetics Corporation is submitting a petition for Determination of Non-regulated Status to the Animal and Plant Health Inspection Service (APHIS) regarding corn with a gene conferring resistance to glufosinate herbicide. This petition requests a determination that glufosinate tolerant line B16 and any progenies derived from crosses between line B16 with other corn lines, and any progeny derived from crosses of line B16 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered a regulated article under regulations in 7 C.F.R. Part 340

Glufosinate (also known as phosphinothricin), the active ingredient in the herbicide Liberty®, controls weeds by inhibiting activity of the enzyme glutamine synthase. Glutamine synthase catalyzes the addition of ammonia (NH_3) to glutamate to form the product glutamine. This enzymatic reaction is the primary mechanism of fixation of nitrogen into organic compounds. Inhibition of glutamine synthase by glufosinate leads to an accumulation of ammonia in the plant and subsequent death due to ammonia toxicity (Tachibana et al., 1986). Glufosinate is a fast acting post-emergence contact herbicide with a wide spectrum of susceptible weeds.

The *bar gene* was isolated from *Streptomyces hygroscopicus*. This gene encodes a phosphinothricin acetyltransferase (PAT) enzyme that converts phosphinothricin to the non-glutamine synthesase inhibiting form, n-acetyl phosphinothricin. A homologous gene, *pat*, has been isolated from *Streptomyces viridochromogenes* and encodes an enzyme with about 85% homology to the *bar* gene product at the amino acid level. The PAT enzyme is highly specific for phosphinothricin and has not been demonstrated to act on naturally occurring amino acids. Hence when introduced into a plant cell PAT will specifically inactivate the herbicidal compound phosphinothricin and consequently normal glutamine synthase activity will be maintained in the plant in the presence of the herbicide.

The corn line for which this determination is requested, B16, contains the *bar* gene. This line was produced by stably inserting the *bar* gene into the genome of an A188 x B73 hybrid cell line. Molecular analysis of B16 indicates that there is a single copy of the *bar* gene and cauliflower Mosaic Virus 35S promoter and two partial copies of the *Agrobacterium tumefaciens* transcript 7 (Tr 7) gene 3′ regulatory region. This line is unaffected by applications of glufosinate as the *bar* gene product inactivates the herbicide.

In corn production, hybrids containing the *bar* gene will allow the farmer to use glufosinate for control of weeds. Glufosinate tolerant corn hybrids will positively impact agronomic practices

in corn by (1) offering the farmer a new, fast acting, wide spectrum weed control option; (2) introducing a new herbicidal mode of action to which no known resistant weeds have developed; (3) increasing flexibility in herbicide applications by allowing applications as needed; (4) reducing dependence on pre-plant and pre-emergence herbicides; and (5) providing an excellent weed control alternative in no-till systems with resultant increases in soil moisture and decreases in erosion and fuel usage.

The corn line B16 has been field tested since 1991 at approximately 26 locations under APHIS field release permits and notifications (see Table VI.1). These field tests indicate that the corn line B16 (1) has no plant pathogenic properties; (2) is unlikely to increase the weediness potential of any cultivated or wild plant species; (3) is no more likely to become a weed itself than the parent inbred lines; (4) does not cause damage of processed agricultural commodities; and (5) is unlikely to harm beneficial organisms. Therefore DEKALB Genetics Corporation requests a determination from APHIS that the corn line B16 and any progenies derived therefrom no longer be considered a regulated article under 7 C.F.R. 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.

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I.Rationale for Development of Glufosinate Resistance Corn

Weeds compete with corn for nutrients, water, and light and when not controlled can significantly reduce the yield of corn. It is especially important to control weed growth early in corn plant development, because even small numbers of weeds can have a dramatic negative impact on crop yield. Weeds are primarily controlled by mechanical or chemical means. Although mechanical cultivation is widely practiced, chemical weed control measures are wide spread and greater than 95% of the corn crop in the United States is treated with chemical herbicides. Indiscriminate use of herbicides, however, can lead to development of resistant weeds. Therefore it is important to develop methods of chemical weed control that represent novel modes of action and are unlikely to select for resistant weeds.

In selecting a herbicide for control of weeds in corn, a chemical must be chosen that has a suitable spectrum of weeds that are killed and will not have adverse long lasting effects on the environment. In addition with increasing no till and minimum till acreage for corn, it is necessary to have weed control agents available that can be applied post-emergence and spot applied as needed. Some of the herbicides currently applied to corn are limited in weed spectrum, may persist in soil or contaminate ground water, or may lead to the development of herbicide resistant weeds. Moreover, some herbicides that have reduced potential for adverse environmental effects and exhibit a broad spectrum of weed killing ability are non-discriminatory in their plant killing ability, i.e. crop plants such as corn are equally affected as weed species. It is only through introduction of genes conferring resistant to such herbicides that these chemicals can be used for weed control in corn.

Glufosinate (also known as phosphinothricin) is a broad spectrum post-emergence herbicide that is rapidly degraded in soil, has a low toxicity to non-target organisms, and does not contribute to ground water contamination. Glufosinate is the active moiety in the *Streptomyces* fermentation product bialaphos (Bayer et al, 1972). The compound inhibits the enzyme glutamine synthase and is believed to kill plants, including corn, as a result of the accumulation of ammonia (Tachibana et al, 1986). Resistance to glufosinate is conferred by the *bar* gene isolated from *Streptomyces hygroscopicus* (De Block et al, 1987). The enzyme phosphinothricin acetyltransferase encoded by the *bar* gene specifically acetylates phosphinothricin at the free amine position and eliminates the glutamine synthase inhibiting activity of glufosinate. Therefore, plants expressing the *bar* gene are resistant to glufosinate.

Glufosinate resistant (GR) corn will permit the use of

glufosinate for weed control in corn. The introduction of glufosinate resistant corn will (1) offer the farmer a new, fast acting, wide spectrum, weed control option; (2) introduce a new herbicidal mode of action to which no known resistant weeds have developed; (3) increase flexibility in herbicide applications by allowing applications as needed; (4) reduce dependence on pre-plant and pre-emergence herbicides; and (5) provide an excellent weed control alternative in no-till systems with resultant increases in soil moisture and decreases in erosion and fuel usage.

The corn line B16 for which a determination of non-regulated status is requested was developed by microprojectile bombardment of embryogenic maize cells. About 12 lines containing the *bar* gene were compared with regards to agronomic performance and resistant to glufosinate. The line B16 was selected for further development based on (1) stability of the trait; (2) a normal pattern of Mendelian inheritance; (3) no observable effects on agronomic performance and resistance to applications of glufosinate up to at least nine times (2.5 lbs active ingredient/acre) the expected field application rate of about 0.28 lb active ingredient/acre. Field trials in 1993 and 1994 indicated that agronomic performance of seven corn hybrids developed from line B16 is similar to the comparable herbicide sensitive hybrid. No unexpected disease or insect interactions were observed in three years of field trials and no evidence of increased weediness was observed in B16 hybrids.

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 (Galinat, 1983): (1) the development of paired female spikelets in corn in comparison to solitary 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 gamily *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 flower 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. Life Cycle of the Crop

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.

E. Hybridization

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. In the United States there is no known species with which corn readily hybridizes. Therefore it is highly unlikely that a gene will spread from corn to a wild species. In addition, for reasons outlined above, even in areas where *Zea* species (e.g. *Z. mexicana, Z. diploperennis*) are present that can cross hybridize with maize, the hybrids are not suitable for cultivation or survival as weeds.

F. Potential for Outcrossing With Wild 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. 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. As discussed above, 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.

III. Description of the Transformation System and Plasmid Utilized

A. Transformation of Corn via Particle Acceleration

The transformation of corn by microprojectile bombardment was described in detail in Gordon-Kamm et al. (1990). Target cells were embrogenic maize suspension cultures. In particular, the B16 transformant was developed from a suspension culture designated SC82 of the genotype A188 x B73. Plasmid DNA containing the *bar* gene was precipitated onto tungsten particles using $CaCl_2$ and spermidine. A suspension of DNA coated microprojectile particles was loaded onto a macroprojectile. The macroprojectile was accelerated by a gunpowder blast. The macroprojectile was stopped by a plastic plate, but the flight of the DNA coated microprojectile particles continued. Particles penetrated target cells, and DNA was released from the particles and integrated into a chromosome of the target cell. Following microprojectile bombardment cells were returned to liquid medium and cultured for 1-2 weeks in the absence of selection. At this time cells were plated on filter papers on medium containing 3 mg/L bialaphos. Filters were transferred weekly to fresh culture medium containing bialaphos. Transformants resistant to bialaphos were identified 6-7 weeks following particle bombardment. Transformants were positively identified by the presence of phosphinothricin acetyltransferase enzyme activity and Southern blot analysis demonstrating the presence of the *bar* gene. Plants were regenerated from transformed callus tissue following transfer of callus to culture medium containing reduced concentrations of auxin hormones and/or cytokinin hormones followed by culture in hormone-free culture medium. Following development of roots, plants were transferred to soil in the greenhouse. Plants were raised to maturity and ears pollinated by the inbred B73. Immature embryos were excised from developing seed 10-20 days following pollination, germinated *in vitro*, and transferred to soil in the greenhouse for further growth. Progeny plants were screened for resistance to glufosinate.

B. Properties of the Non-transformed Hybrid A188 x B73

Zea mays hybrid A188 x B73 is the genotype that was genetically modified to be resistant to the herbicide glufosinate. This genotype was selected based on the ability to establish and maintain callus and suspension cultures of A188 x B73 tissue that are capable of regeneration of fertile plants (Kamo and Hodges, 1986). A188 is a non-commercial inbred that was recognized for its amenability in tissue culture. B73 is a stiff stalk inbred that has been used extensively in commercial corn hybrids. Once a desirable transformant source has been identified in the A188 x B73 genotype, the source is backcrossed to elite inbreds for the purpose of producing commercial hybrids. A single transformant may be backcrossed into a wide range of genotypes. For example,

the B16 transformant source has been backcrossed into over 50 different elite inbreds from which it is possible to produce a large number of commercial hybrids with relative maturities ranging from 85 to 115 days.

C. Construction of the Plasmid Utilized for Transformation, pDPG165.

pDPG165 is a plasmid vector containing a 35S promoter- *bar* structural gene - *Agrobacterium tumefaciens* transcript 7 3'region expression cassette that is introduced into corn cells using microprojectile bombardment. The *bar* expression cassette is inserted in the high copy number *E.coli* plasmid pUC19 (Yanisch-Perron et al., 1985). A gene encoding ampicillin resistance (*bla*) is present on this plasmid for selection and maintenance of the plasmid in *E. coli*. This gene is under control of a bacterial promoter and it is highly unlikely that it would express in a plant cell.

A map of plasmid pDPG165 is provided in Figure III.1. A description of each of the elements of the vector is included in Table III.1.

Table III.1. Summary of Sequences Present in Plasmid pDPG165

Vector Component	Size, Kb	Description
35S	0.80	The Cauliflower Mosaic Virus (CaMV) promoter (Odell et al., 1985)
bar	0.57	The *bar* gene encoding phosphinothricin acetyltransferase isolated from *Streptomyces hygroscopicus* (White et al., 1990).
Tr7	0.60	The untranslated 3' region from *Agrobacterium tumefaciens* T-DNA transcript 7 (Dhaese et al., 1983)
lac	0.24	A partial lacI coding sequence, the promoter plac, and a partial coding sequence for β-galactosidase or lacZ protein (Yanisch-Perron et al., 1985)
bla	0.86	The TEM type β-lactamase gene 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.
ori-pUC	0.65	The origin of DNA replication from the *E. coli* high copy plasmid pUC19 (Yanisch-Perron et al., 1985)

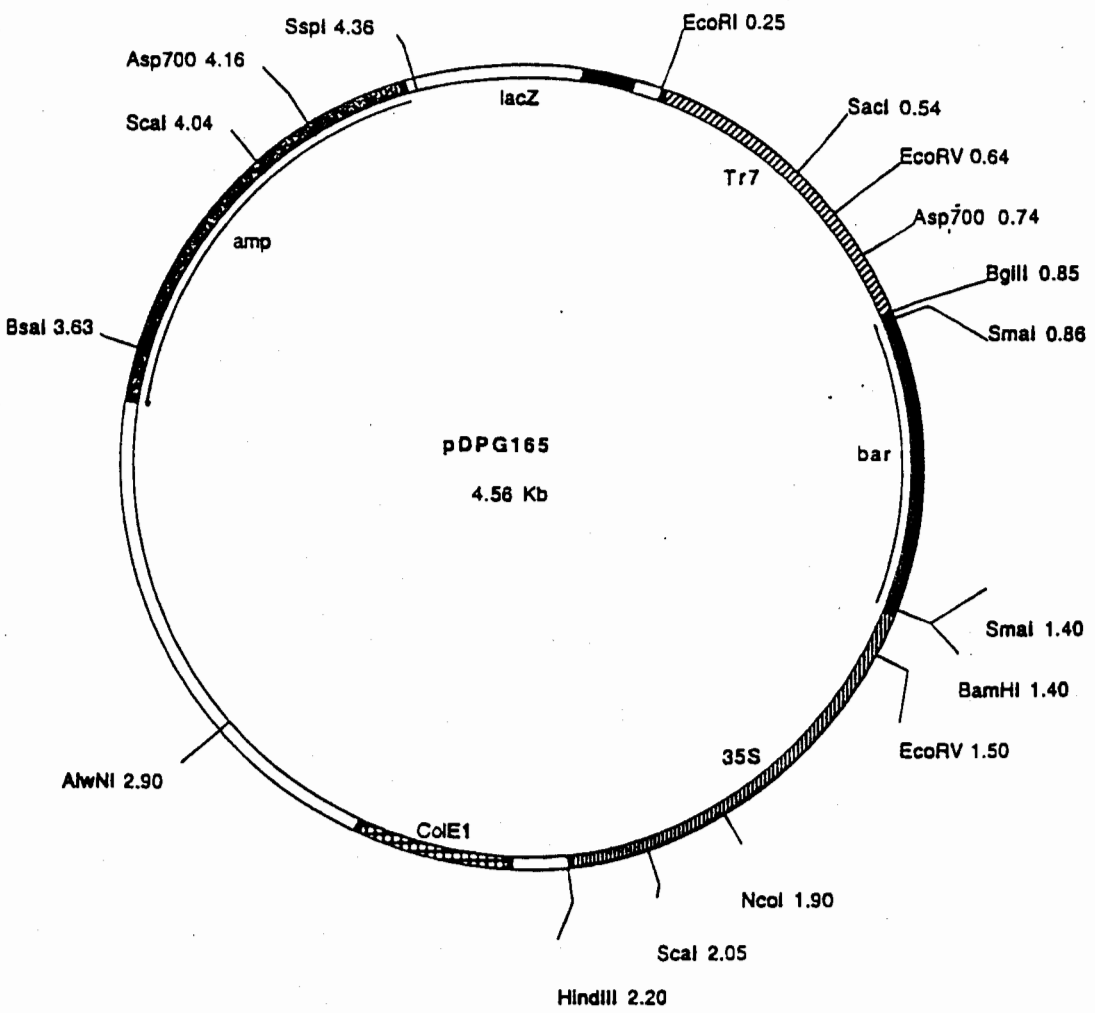


Figure III.1. Plasmid Map of Vector pDPG165.

IV. Open Reading Frames in pDPG165

A. *bar* Gene

The herbicide glufosinate is an inhibitor of the enzyme glutamine synthase. In the presence of phosphinothricin, sensitive cells do not fix ammonia through the glutamine synthase pathway and cell death due to ammonia toxicity follows (Tachibana et al., 1986). Expression of the *bar* gene in microogranisms or plants confers phosphinothricin resistance on those cells or plants. Maize plants resistant to phosphinothricin were produced by stably inserting the *bar* gene into the chromosome of corn. Expression of the *bar* gene leads to inactivation of phosphinothricin and confers on those maize plants a herbicide resistant phenotype.

The *bar* gene introduced into line B16 encodes the enzyme phosphinothricin acetyltransferase (PAT). The *bar* gene was isolated from the soil microorganism *Streptomyces hygroscopicus* (ATCC 21705). In this organism, the *bar* gene product may play a biosynthetic as well as a degradative function (Thompson et al., 1987). *Streptomyces hygroscopicus* produces the antibiotic compound bialaphos (phosphinothricin alanyl alanine). The PAT enzyme catalyzes acetylation of demethyl phosphinothricin, an intermediate step in the biosynthesis of bialaphos. The second enzymatic function of PAT is the acetylation of phosphinothricin, and inactivation of its herbicidal activity. In *Streptomyces hygroscopicus* this function serves to protect the organism from the toxicity of the antibiotic that it produces.

A gene similar to *bar* has been identified in *Streptomyces viridochromogenes* and designated *pat*. Similar to *S. hygroscopicus*, *S. viridochromogenes* produces the antibiotic bialaphos. However, unlike *S. hygroscopicus*, *S. viridochromogenes* is sensitive to bialaphos and phosphinotricin. A gene (*pat*) encoding an enzyme with PAT activity was isolated from *S. viridochromogenes* following selection of phosphinothricin resistant mutants (Strauch et al, 1988; Wohlleben et al, 1988) In contrast to *S. hygroscopicus*, *S. viridochromogenes* synthesizes bialaphos, but is not naturally resistant to its activity. The amino acid sequences of *bar* and *pat* are 85% homologous with most of the differences occuring in the carboxy and amino termini of the proteins. Furthermore, there is 87% homology in the nucleic acid sequences between the *pat* and *bar* genes. Neither *S. hygroscopicus* and *S. viridochromogenes* are known to be plant pests.

The *bar* gene is comprised of about 549 base pairs encoding a protein (phosphinothricin acetyltransferase) of 183 amino acids with a molecular weight of about 21,000. The gene has been completely sequenced (White et al., 1990). The GTG initiation codon present in the *S. hygroscopicus* gene was mutated to ATG to

conform with plant codon usage. The *bar* gene was cloned from *Streptomyces hygroscopicus* ATCC 21705. This strain of *Streptomyces* was collected in Japan. The PAT enzyme catalyzes the transfer of an acetyl group from acetyl CoA to the amino group of phosphinothricin. The enzyme is highly substrate specific (Thompson et al., 1987). The substrate affinity for phosphinothricin is more than 30 times higher than the affinity for demethylphosphinothricin (the biosynthetic pathway intermediate) and over 300 times higher than the affinity for the amino acid glutamate. It is therefore highly unlikely that PAT will acetylate any naturally occuring compound in maize cells.

B. Non-coding Regulatory Regions Controlling the *bar* gene

The *bar* gene expression unit contained in pDPG165 comprises a Cauliflower Mosaic Virus 35S promoter, the *bar* gene and the *Agrobacterium tumefaciens* transcript 7 3′ region (Tr7). The 35S promoter was chosen for expression in maize, because it was believed to confer high level expression in many tissue types in the plant. The 35S promoter has been described in detail by Odell et al. (Odell et al., 1985). The 35S promoter present in pDPG165 is an approximately 800 base pair sequence described by Baulcombe et al. (1986) in the vector pROK1. The 35S promoter DNA sequence and development of the pROK1 35S promoter is described in Franck et al., 1980, Guilley et al., 1982, and Bevan et al., 1985.

The 3′ region associated with the *bar* gene in pDPG165 is a 534 bp DNA sequence derived from transcript 7 of the *Agrobacterium tumefaciens* T-DNA (T-DNA open reading frame 3). The DNA sequence and position of the polyadenylation signal are described in Dhaese et al. (Dhaese et al., 1983). The polyadenylation signal sequence is at positions 137-142 of the Tr7 element present in pDPG165.

Although the promoter and 3′ regulatory regions that control expression of the *bar* gene were derived from organisms considered to be plant pests, neither sequence contains protein coding sequences from these organisms and the regulatory region sequences alone cannot confer plant pest status on the recipient plant.

C. *bla* Gene Encoding β-lactamase

The plasmid vector pDPG165 contains the *E. coli* gene encoding β-lactamase derived from the parent plasmid pUC19 (Yanisch-Perron et al., 1985). In the B16 integration event the β-lactamase gene is truncated as evidenced by deletion of a restriction site and DNA sequence analysis (section V.C.2). In order to determine whether the β-lactamase gene present in B16 encoded a function enzyme, the gene was cloned from B16 into an *E. coli* plasmid vector lacking a β-lactamase gene, but containing a selectable

marker gene encoding neomycin phosphotransferase II. *E. coli* clones were recovered containing the B16 β-lactamase gene. These clones were not resistant to ampicillin and it was, therefore, concluded that the β-lactamase gene in B16 was truncated and encodes a non-functional β-lactamse protein. As the β-lactamase gene is not functional in *E. coli*, it it believed that the gene is not functional in plant cells. In addition, even if the β-lactamase gene in B16 were full length, the gene is controlled by prokaryotic (*E. coli*) transcription and translation signals and would, therefore, not be expected to be expressed in maize.

V. Genetic Analysis and Molecular Characterization of the B16 Line

A. Description, History, Mendelian Segregation and Genetic Mapping of B16

The B16 transformant was recovered as a bialaphos resistant cell line following particle bombardment of a suspension culture, designated SC82, initiated from A188 x B73 type II callus line. The isolation of this transformant is described in detail in Gordon-Kamm et al., 1990. Progeny of this transformation event were maintained and characterized in the greenhouse throughout 1990. In March, 1991, backcross conversions of B16 to elite inbreds were initiated in the field in Kihei, Maui, Hawaii (USDA APHIS permit #90-332-02). Since that time backcross conversions to elite inbreds at proceeded at three to four generations per year (USDA APHIS numbers in Table VI.1). Backcross conversions of some inbreds were completed in 1993, and finished glufosinate resistant maize hybrids were analyzed in the field in 1994. Table V.1 documents genetic segregation of glufosinate resistance in backcrosses of heterozygous B16 plants to elite inbreds. This data represents backcrosses of 32 elite inbreds to the B16 line from 1990 through 1993. Chi squared analysis for homogeneity of the data from all tests gives a χ^2 value of 0.851 indicating that the data is homogenous and can be combined. Chi squared analysis of the summed data indicates that segregation for glufosinate resistance is 1:1 as expected in backcrosses of heterozygous plants of the B16 line to elite inbreds.

Table V.1. Segregation for Glufosinate Resistance in Backcrosses of B16 to Elite Inbreds

Testing Date	Resistant Plants	Sensitive Plants	χ^2
1990	18	18	0
1991	238	247	0.13
1992	87	83	0.05
1993	753	710	1.21
TOTAL	1,096	1,058	0.636

As will be demonstrated below, the B16 integration event is a single copy integration of the *bar* gene in the maize genome. The insertion site was mapped by two techniques. First, the B16 integration site was mapped to the long arm of chromosome 3 by use of waxy translocations *wx*09A and *wx*08A (Laughnan and Gabay-Laughnan, 1994). Second, the maize genomic sequence flanking the

B16 integration event was cloned and the maize DNA sequences flanking the plasmid insertion site mapped to the long arm of chromosome 3 using RFLP mapping of a recombinant inbred population (Burr et al., 1994) in close proximity to RFLP markers *npi91A*, *npi257*, and *bnl17.27*.

The combination of genetic segregation and genetic mapping data demonstrates that the B16 transformant contains a stable integration of pDPG165 DNA sequences. Furthermore this integration event has remained stably integrated in the maize genome for over 10 generations.

B. DNA Analysis of Glufosinate Resistant Corn Line B16

As described above, the maize line B16 was produced by particle bombardment of an A188 x B73 suspension culture with the plasmid pDPG165. DNA analysis was conducted to characterize the B16 integration event with respect to the number of insertion sites, plasmid copy number in the maize genome, and the identity of elements from pDPG165 that were integrated in B16.

1. Number of insertion sites in line B16

The number of pDPG165 insertions in B16 was demonstrated following digestion of genomic B16 DNA with a restriction enzyme that does not cut the plasmid vector. Upon probing of a Southern blot with the entire pDPG165 plasmid, one would expect a single band on a Southern blot for each insertion site. B16 genomic DNA was restriction enzyme digested with *NsiI* for which there are no cleavage sites in pDPG165. A Southern blot of this *NsiI*-digested B16 DNA was probed with pDPG165 and a single band of about 8 Kb was observed (Figure V.1(a)). This results confirms the observation that there is a single insertion of pDPG165 in B16.

Southern blot analysis was used to determine the number of plasmid insertion events in line B16. Genomic DNA isolated from B16 was cut with the restriction enzymes *NcoI* or *BglII* and probed in a Southern blot with a pDPG165 SmaI fragment that comprises the *bar* coding sequence. Each of these restriction enzymes cuts once in the plasmid pDPG165, and would be expected to produce a unique Southern blot hybridization pattern when probed with *bar*, because *NcoI* or *BglII* cut once in the introduced plasmid and the second restriction enzyme site will be variable as it is located in flanking maize genomic DNA. If a single copy of *bar* integrated into the maize genome in the B16 line, *NcoI* or *BglII* digests of B16 DNA should yield a single *bar*-hybridizing DNA fragment, consisting of a hybrid fragment of plasmid and plant DNA. A single *bar* hybridizing band was observed using *NcoI* or *BglII*, indicating a single integrated copy of *bar* (Figure V.1(b)).

The number of copies of pDPG165 at the single insertion site was estimated in comparisons to copy number reconstructions wherein

plasmid DNA was present on a Southern blot in an amount that was equivalent, on a molar basis, to a single copy of that sequence in the maize genome. The Southern blots of *BglII* or *NcoI* digested B16 genomic DNA probed with the *bar* gene, shown in Figure V.1(b), suggested that there is a single copy of the *bar* gene present at the pDPG165 insertion site in the B16 line.

Based on restriction enzyme digestions and Southern blotting, and the genetic mapping and segregation data discussed above, it is concluded that there is a single insertion event of pDPG165 in the B16 genome and it is further suggested that there is a single copy of the *bar* gene at the insertion site. Detailed restriction enzyme mapping and Southern blotting of the B16 insertion described below confirms these conclusions.

2. Analysis of pDPG165 Sequences Integrated into the Corn Genome in the B16 line

Copy number and genetic mapping results suggested that there was a single insertion event of pDPG165 in the B16 line. Southern blot analysis were used to further characterize the DNA sequences inserted in the B16 line.

Gordon-Kamm et al. (1990) demonstrated that a *HindIII/EcoRI* digest of B16 callus or R_0 DNA, probed with *bar*, yielded a fragment of approximately 3 kb (B16 was refered to as "E2/E5" in Gordon-Kamm et. al., 1990) Unique *HindIII* and *EcoRI* sites flank the *bar* expression cassette in pDPG165 (Figures III.1, V.7(a)), and digestion of pDPG165 with *EcoRI/HindIII* releases a fragment of 1.9 kb containing *bar*. This indicated that either the *HindIII* site or the *EcoRI* site, or both, were missing or rearranged with respect to *bar* in the plasmid DNA that integrated in B16. B16 and pDPG165 DNA were digested with *BamHI/EcoRI* or *BglII/HindIII* and probed with *bar*. Comparing the size of *bar*-hybridizing restriction fragments generated from B16 genomic DNA with those derived from pDPG165 demonstrated that the genomic fragments were significantly larger than the plasmid fragments. This analysis indicated that both the *HindIII* and *EcoRI* sites that flank the *bar* expression cassette in pDPG165 were not cut in B16, or were missing, or were rearranged as to their original orientation to *bar* in pDPG165. These data suggested that the pDPG165 insertion in the B16 line involved some rearrangements, duplications, or deletions of plasmid DNA.

Southern blot analysis was used to determine the presence, and orientation relative to *bar*, of the 35S promoter, Tr7 3' region, β-lactamase gene, and pUC origin of DNA replication.

a. 35S promoter

pDPG165 contains an 800 base pair *BamHI/HindIII* fragment comprising the 35S promoter (Baulcombe et al., 1986). Genomic

DNA isolated from the B16 line was digested with *NsiI* and *BamHI*. The pDPG165 insertion in the B16 lies on an 8 Kb maize genomic DNA *NsiI* fragment (Figure V.1(a)). *BamHI* cuts pDPG165 at a single site between the 35S promoter and *bar* gene. *NsiI/BamHI* digested B16 genomic DNA was probed with a *BamHI/HindIII* fragment from pDPG165 that comprises the 35S promoter and a single 3 Kb fragment hybridized indicating that at least part of the 35S promoter was present.

Because the unique *HindIII* site that lies at the 5' end of the 35S promoter in pDPG165 is absent in the B16 insertion, a portion of the 5' end of the promoter must be absent in the B16 line. Therefore genomic DNA of the B16 line and plasmid pDPG165 were digested with *BglII/NcoI* or *BglII/ScaI* and probed with the *SmaI* fragment comprising the *bar* gene from pDPG165. Comparisions of the size of *bar* hybridizing fragments in genomic B16 and plasmid DNA digests on Southern blots indicated that the 35S promoter is present in the B16 at least up to and including the ScaI restriction enzyme site (Figure V.2). Therefore, up to 100 base pairs of the 5' end of the 800 base pair 35S promoter present in pDPG165 was not inserted in the maize genome in the B16 line.

b. *bar* gene

The *bar* gene in pDPG165 is a 549 base pair sequence contained within a 569 base pair *SmaI* fragment derived from pIJ4104 (White et al., 1990). Gordon-Kamm et al. (1990) demonstrated that *SmaI* digestion of B16 genomic and pDPG165 plasmid DNA produced identical, expected, approximately 600 base pair bands on a Southern blot probed with the *SmaI* fragment from pDPG165. Furthermore, digestion of B16 genomic DNA with *BamHI* and *BglII* also produced the expected band of about 600 base pairs on a Southern blot when probed with the *SmaI* fragment from pDPG165. Thus the intact *bar* gene is present in the B16 line.

c. Tr7 3' Region

Genomic DNA from the B16 line was cut with *NsiI/BamHI*. A Southern blot of digested B16 DNA probed with a *EcoRI/BglII* fragment of pDPG165 comprising the Tr7 3' region showed two hybridizing bands, at 5 and 3 Kb, indicating that there is a copy of Tr7 at the expected position 3' of the *bar* gene (the 5 Kb *NsiI/BamHI* fragment), but a second copy was present on the 3Kb *NsiI/BamHI* fragment that hybridized to 35S. As described above the expected 1.8 Kb *EcoRI/HindIII* band observed in pDPG165 is not present in the B16 line due to alterations in both the *EcoRI* and *HindIII* sites. Therefore, the copy of the Tr7 3' region that is positioned 3' to the *bar* gene is incomplete in that the *EcoRI* site at the 3' end of the element is absent. Genomic DNA from the B16 line and pDPG165 were digested with *BamHI* in combination with *Asp700*, *EcoRV*, *SacI*, or *EcoRI*. A Southern blot of these restriction digests was probed with the 0.6 Kb *SmaI bar* fragment

from pDPG165. Comparison of the *bar*-hybridizing fragments in these restriction digests indicated that the end of the B16 insertion lies in the 215 base pairs between the *SacI* and *EcoRI* sites in Tr7 (Figure V.3). DNA sequence analysis (Appendix 1) indicates that this copy of Tr7 is truncated 1 base pair 5′ of the *EcoRI* restriction site that is present in pDPG165, and therefore 526 base pairs of the Tr7 sequnce are present. As the polyadenylation signal sequence is located between the *Asp700* and *EcoRV* sites in Tr7 (Dhaese et al., 1983), this site is present in the B16 line.

A second partial copy of Tr7 is located 5′ of the 35S promoter in the B16 line (Figure V.7(a)) and in an opposite orientation to the copy located 3′ of the *bar* gene. This copy of Tr7 was cloned from the B16 line on a *BamHI/NsiI* fragment and restriction mapping has demonstrated that the *EcoRI*, *SacI*, EcoRV and *Asp700* sites present in Tr7 are present in this copy of Tr7 in the B16 line. DNA sequence analysis (Appendix 1) indicates that 476 base pairs of the 527 base pair Tr7 DNA element is present in the copy of Tr7 located 5′ of the 35S promoter in the B16 line. Therefore, the polyadenylation signal present between the *EcoRV* and *Asp700* restriction sites is present in this copy of Tr7. . The presence of duplicate copies of Tr7 in B16 that overlap in DNA sequence, e.g., presence of *SacI* and *EcoRV* sites, suggests that the two copies of Tr7 present in the B16 line arose from two different molecules of pDPG165 (Appendix 1).

d. β-Lactamse Gene

The end of the pDPG165 insert 5′ to the *bar* gene in the B16 line lies within the β-lactamase gene. This end was delineated by comparing restriction enzyme digests of genomic DNA from the B16 line and pDPG165. DNA was digested with *EcoRI* in combination with *SspI*, *Asp700*, *ScaI*, *BsaI*, or *AlwNI* and a Southern blot probed with the *EcoRI/SspI lacZ* fragment (Figure V.4). Genomic B16 DNA and plasmid restriction digests up to including the *ScaI* site in the β-lactamase gene demonstrate identical bands. Although the *EcoRI/BsaI* fragment appears to be identical in digested B16 genomic DNA and pDPG165, DNA sequence analysis indicates that the *BsaI* site in the B16 digest actually lies in maize genomic DNA and not in a pDPG165 derived sequence (see DNA sequence in Appendix 1). The absence of the *BsaI* and *AlwNI* sites suggests that the pDPG165-maize genomic DNA junction lies somewhere in the β-lactamase gene between the *BsaI* and *ScaI* sites. The *ScaI* and *BsaI* sites are located 305 and 716 base pairs respectively from the beginning of the the β-lactamase gene, suggesting that only this portion of the β-lactamase gene is present in the B16 line. Furthermore, DNA sequence analysis of the 3′ end of the β-lactamse gene that was cloned from the B16 line demonstrated that the gene is truncated at base pair 568 of the 858 base pair coding sequence of the mature β-lactamse gene in the B16 line.

e. ori-pUC

The *AlwNI/HindIII* fragment from pDPG165 containing the pUC origin of DNA replication did not hybridize to B16 DNA. *NsiI/BamHI* digested genomic DNA from the B16 line was probed on a Southern blot with the *AlwnI/HindIII* fragment and no hybridizing fragment was observed (Figure V.5). The pUC origin of DNA replication was detected in Southern blots of pDPG165. Therefore, the pUC origin of DNA replication is not present in the B16 line.

f. Stability of the DNA Insert in the B16 Line

The stability of the DNA insertion in the B16 line was assayed by genetic and molecular assays. Genetic segregation throughout generations has remained constant at the expected Mendelian ratios. Furthermore, genetic mapping of the insertion site by waxy translocations in 1993 and RFLP mapping of the insertion site in 1995 demonstrated a single insertion site on the long arm of chromosome 3.

Stability of the B16 insertion is further evidenced by Southern blot data. Genomic DNA from an R_1 plant of the B16 line and a corn hybrid containing the B16 insertion (11 generations later) were compared on a Southern blot following digestion with *NsiI* or *NsiI/BamHI* and probing with the pDPG165 *SmaI bar* gene fragment (Figure V.6). In both generations the B16 insertion is present on an 8 Kb *NsiI* fragment. The *bar* DNA sequence is also present on a 5 Kb *NsiI/BamHI* fragments in both generations. Thus, it is suggested that the B16 insertion event in genomic DNA of an R_1 plant is equivalent to the insertion event in a later generation plant and, therefore, the B16 insertion is stably integrated in the maize genome.

g. Summary of the DNA Analysis of the B16 Line

The DNA insert in the B16 line is shown schematically in Figure V.7(a) and comprises about 3500 base pairs derived from the 4562 base pair plasmid pDPG165 inserted at a single site in the corn genome. Figure V.7(b) shows the plasmid pDPG165 origin of each of the DNA segments present in the B16 line. The entire insertion is contained on a maize genomic *NsiI* fragment of approximately 8 kb. The insertion contains a single intact copy of the *bar* gene, and single incomplete copies of the 35S promoter and β-lactamase gene. The insert contains two partial copies of the Tr7 3'-end. The insertion lacks hybridization to a pDPG165 fragment containing the ColE1 origin of replication. One end of the DNA insert in the B16 line is located in the Tr7 3' element located 3' of the *bar* gene between the *SacI* and *EcoRI* sites of Tr7 at the 3' end of the element. The other end of the DNA insert in the B16 line is located in the β-lactamase gene between the *ScaI site* and the end of the gene. There is no copy of the pUC origin of DNA replication in the B16 line.

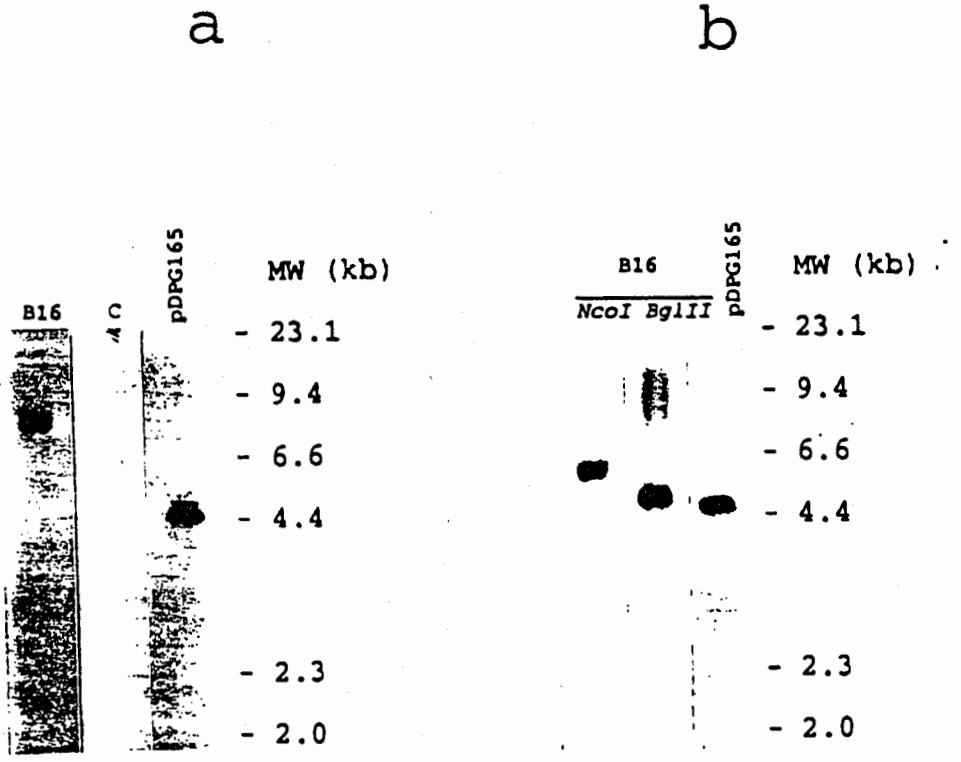


Figure V.1. **Southern Blot Analysis of Copy and Insertion Number in Transgenic Line B16.** (a): Approximately 2.5 µg of B16 DNA (B16) was digested with *NsiI* and probed with ^{32}P-labelled pDPG165. *EcoRI*-digested DNA (2.5 µg) from a nontransformed plant served as a control (C). Lane designated pDPG165 contained *EcoRI*-digested pDPG165 loaded to approximate one copy per maize genome. (b): Approximately 2.5 µg of genomic DNA isolated from the B16 line (B16) was used for analysis. B16 DNA was digested with *NcoI* or *BglII*. Digests were probed with ^{32}P-labelled *bar* probe (0.6 kb *SmaI* fragment from pDPG165). The lane designated pDPG165 contained *EcoRI*-digested pDPG165 loaded to approximate one copy per maize genome.

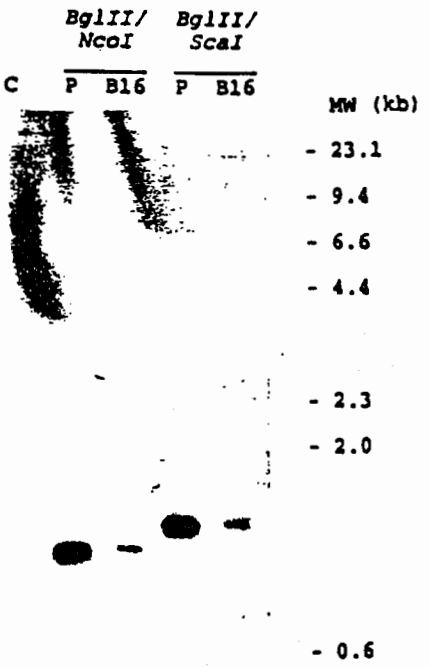


Figure V.2. **Southern Blot and Restriction Digest Mapping of the 35S Promoter in Transgenic Line B16.** Approximately 2.5 µg of genomic B16 DNA (B16) or 20 pg of plasmid pDPG165 (P) were digested with *BglII* in combination with *NcoI* or *ScaI* and probed with ^{32}P-labelled *bar* probe (0.6 kb *SmaI* fragment from pDPG165). Approximately 2.5 µg of nontransformed maize genomic DNA digested with *BamHI/BglII* was used as a control.

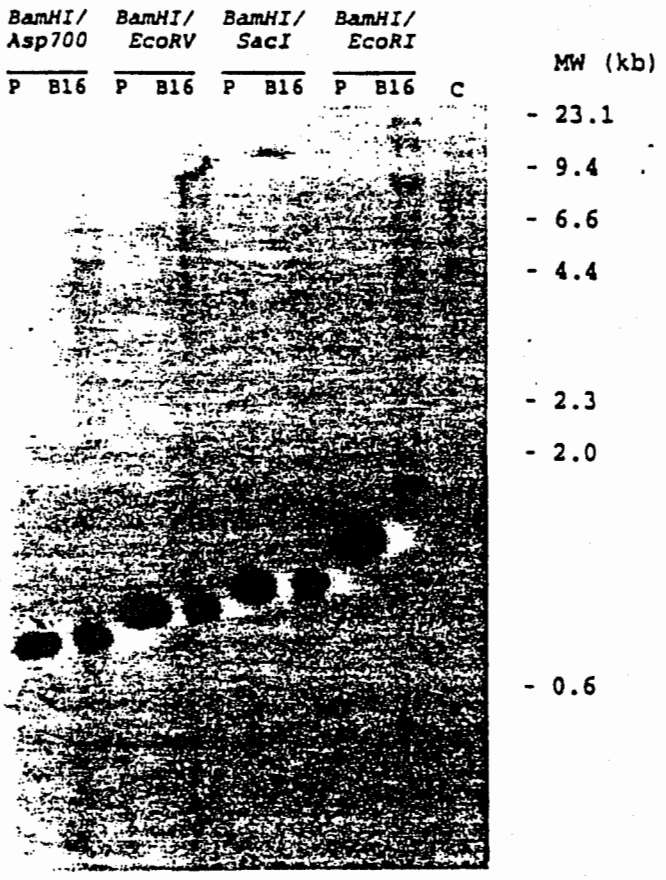


Figure V.3. **Southern Blot and Restriction Digest Mapping of the Tr7 3' region in Transgenic Line B16.** Approximately 2.5 µg of genomic B16 DNA (B16) or 20 pg of plasmid pDPG165 (P) were digested with *BamHI* in combination with *Asp700, EcoRV, SacI,* or *EcoRI* and probed with ^{32}P-labelled *bar* probe (0.6 kb *SmaI* fragment from pDPG165). Approximately 2.5 µg of nontransformed maize genomic DNA digested with *BamHI/EcoRI* was used as a control.

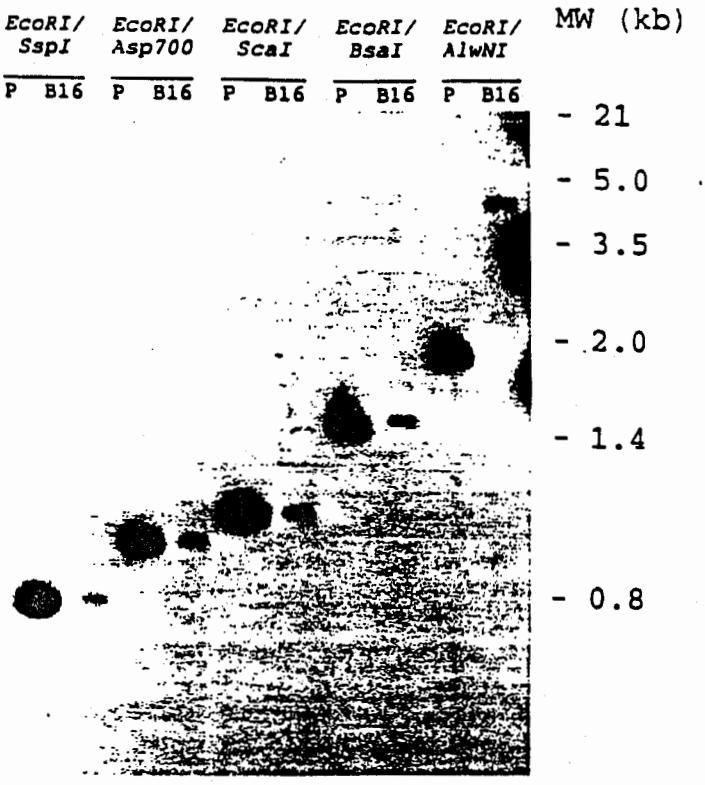


Figure V.4. **Southern Blot and Restriction Digest Mapping of the β-Lactamase Gene in Transgenic Line B16.** Approximately 2.5 µg of genomic B16 DNA (B16) or 20 pg of plasmid pDPG165 (P) were digested with *EcoRI* in combination with *SspI, Asp700, ScaI, BsaI,* or *AlwNI* and probed with the lacZ region from pDPG165 (^{32}P-labelled 0.6 kb *EcoRI/SspI* fragment from pDPG165).

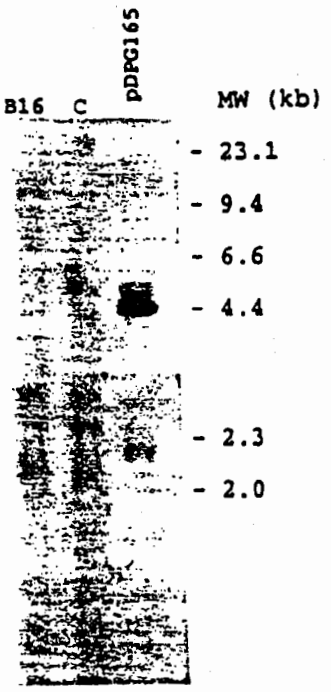


Figure V.5. **Southern Blot Analysis of the Prokaryotic Origin of Replication in Transgenic Line B16.** Approximately 2.5 µg of genomic B16 DNA (B16) or DNA from a nontransformed control plant (C) were digested with *NsiI/BamHI* and probed with a ^{32}P-labelled fragment from pDPG165 containing the ColE1 origin of replication (0.8 kb *AlwNI/HindIII* fragment). Lane designated pDPG165 contained *EcoRI*-digested pDPG165 loaded to approximate one copy per maize genome.

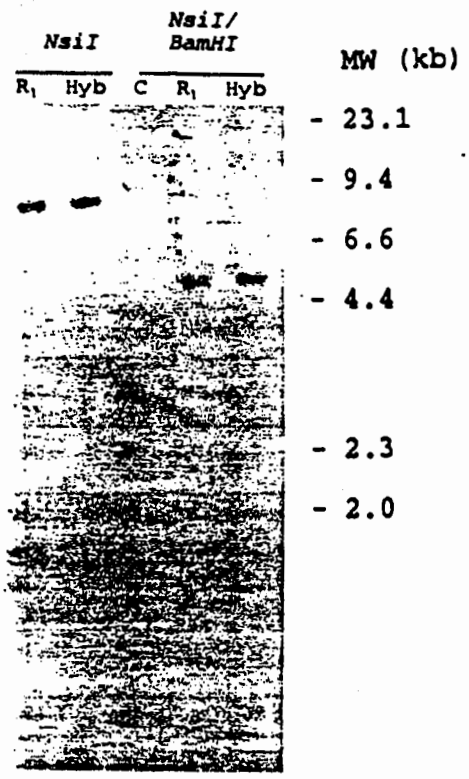
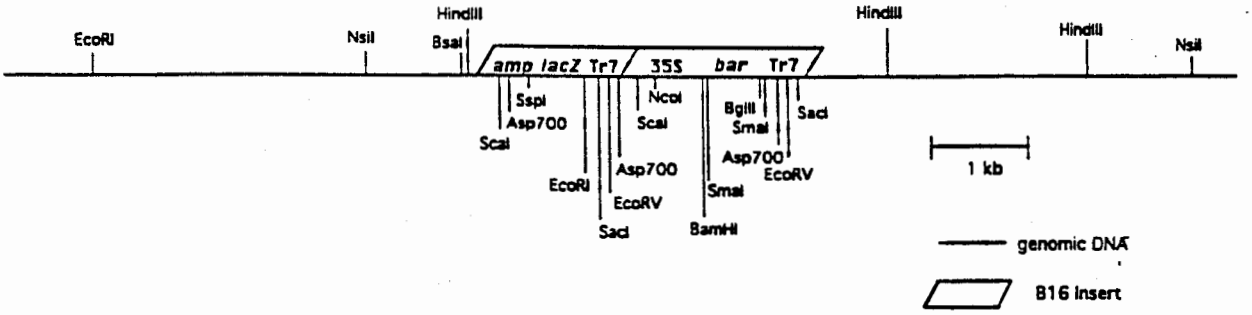


Figure V.6. **Southern Blot Analysis to Determine the Stability of the B16 Insertion.** Approximately 2.5 µg of genomic DNA from a B16 R_1 plant (R_1), a B16 hybrid plant (Hyb), or a nontransformed control (C) were used for analysis. The DNA was digested with either *NsiI* alone or in combination with *BamHI* and probed with ^{32}P-labelled *bar* probe (0.6 kb *SmaI* fragment from pDPG165).

a



b

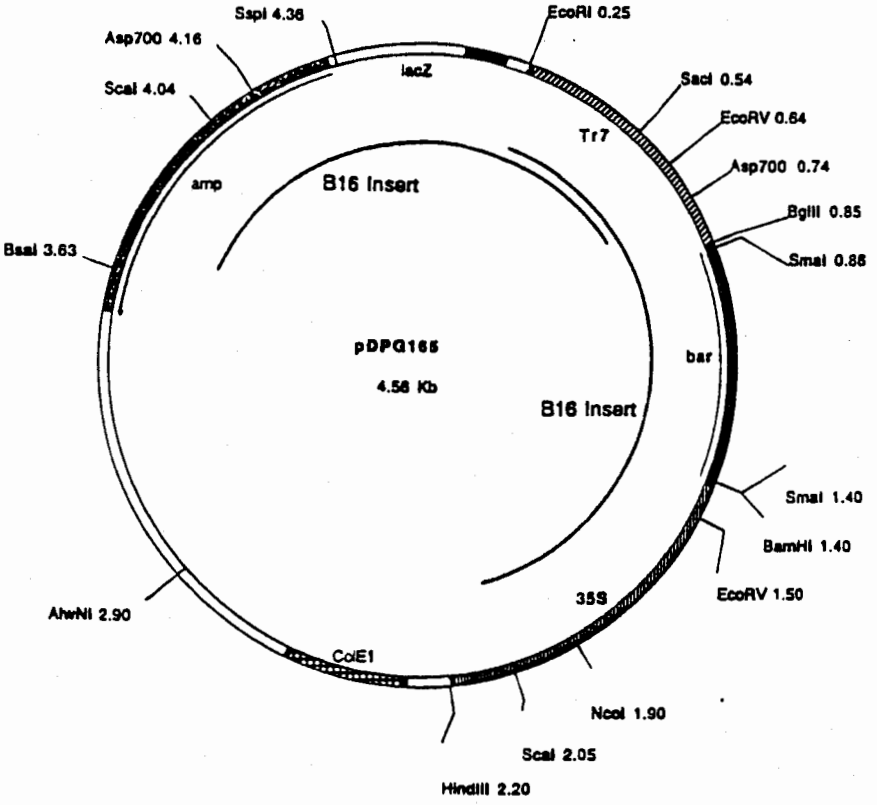


Figure V.7. **Schematic Representation of the B16 Insertion.** (a): Restriction map of the B16 insertion and flanking maize genomic DNA. (b): Restriction map of plasmid pDPG165 and designation of the regions of the plasmid that integrated into B16.

C. Expression of Introduced Genes in the B16 Line

Expression of the *bar* gene and the β-lactamase gene was analyzed in the B16 line. The levels of PAT protein were determined in coleoptile, leaf, root, cob, stalk, husk, pollen, silk, silage and seed. PAT protein was detected on Western blots using polyclonal antibody and visualized by a chemisluminecense assay. Autoradiograms were scanned and PAT protein amounts calculated based on purified PAT protein present on each Western blot. Expression of the β-lactamase gene was determined in the B16 line as well as in *E. coli* following cloning of the β-lactamase gene from the B16 line into *E.coli*. Functionality of the β-lactamase gene present in the B16 line was assayed in *E. coli*. Furthermore, Western blot analysis using a commercially available polyclonal antibody was used to determine the presence of β-lactamase in plants of the B16 line and in an *E. coli* strain containing the β-lactamase gene isolated from the B16 line.

1. Expression of the *bar* Gene

PAT protein levels were determined in crude protein extracts isolated from various plant tissues by Western blot. Proteins were separated on polyacrylamide gels and blotted to nitrocellulose membranes. Ten to 250 micrograms of extracted maize protein from each sample were loaded on a polyacrylamide gel. The amount of protein run on a gel varied and depended on the level of expression of the PAT protein in a particular tissue. Six to seven purified PAT protein standards in amounts from 10 nanograms to 200 nanograms are present on each Western blot. PAT protein was detected with an anti-PAT polyclonal antibody produced in rabbits in combination with anti-rabbit IgG-peroxidase and chemiluminescent detection using the Enhanced Chemiluniscent System (ECL) from Amersham Corporation (Arlington Heights, IL). Autoradiograms were scanned and protein amounts determined using Whole Band Analysis with Bio Image software on a Sun SPARCstation IPC computer (hardware and software package supplied by Millipore Corporation, Bio Image Products, Ann Arbor, MI). Standards were plotted using Sigma Plot (Jandel Scientific, San Rafael, CA) and linear regression analysis and interpolation of experimental values were determined using Lotus 123 (Lotus Devleopment Corporation, Cambridge, MA). Under the conditions employed, 10 nanograms or more of PAT protein could be quantitated. Although less than 10 nanograms of PAT could be visualized, scanning and quantitation of amounts could not be accurately accomplished. No band was present on Western blots of control untransformed tissue corresponding to the approximately 22 Kd molecular weight of PAT protein.

PAT levels were consistent in six hybrid maize lines into which the B16 transformation even had been introgressed. These hybrids had relative maturities from 88 days to 114 days and contained

from 0.5 - 1 ng of PAT per μg of total soluble protein extracted from leaves. These results suggested that there is no genotype effect on expression of the *bar* gene. Expression analysis of the *bar* gene in various maize tissues is summarized in Table V.2. PAT protein levels were highest in vegetative tissues. PAT levels detected in coleoptile, leaf, root, prop root, stalk, cob, and husk were in the range of 1 - 4.6 ng PAT per μg of total soluble protein. This range corresponds to 0.1 - 0.46% of total soluble protein. PAT levels determined in a developmental analysis of F_2 seed ranged from 0.8 ng/μg total soluble protein in unfertilized ovules to less than 0.02 ng/μg of total soluble protein in mature seed. PAT protein concentrations in F_1 hybrid seed, as would be sold to the farmer as seed corn, were slightly higher, 0.06 ng/μg total soluble protein, than the levels observed in F_2 seed (0.02 ng/μg total soluble protein), as would be harvested by the farmer as grain product. The concentration of PAT protein in kernels was over ten-fold lower than the concentration in leaf. No PAT protein was detected in pollen or silks. Silage was produced from the B16 line and no PAT protein was detected in that silage.

Table V.2. PAT Protein in Various Tissues from the B16 Line

Tissue (age)	PAT Concentration (ng/μg total	PAT Concentration (ng/mg fresh weight)
Coleoptile (6 days)	1.8 ng/μg	13.8 ng/mg
Leaf (24 days)	1.0 ng/μg	55.6 ng/mg
Leaf (44 days)	2.8 ± 0.1 ng/μg	166.0 ± 24.2 ng/mg
Leaf (93 days)	2.1 ng/μg	106.1 ng/mg
F_2 ovule (0 days pp)	0.8 ng/μg	5.5 ng/mg
Immature F_2 seed (16 days pp)	0.3 ng/μg	3.4 ng/mg
Immature F_2 seed (27 days pp)	0.3 ng/μg	5.7 ng/mg
Mature F_2 seed (45 days pp)))	0.02 ng/μg	1.9 ng/mg
Hybrid seed (F_1)	0.06 ng/μg	4.2 ng/mg
Root (24 days)	1.3 ng/μg	8.1 ng/mg
Poot (44 days)	1.9 ± 0.3 ng/μg	19.8 ± 2.4 ng/mg
Prop root (49 days)	2.4 ng/μg	67.2 ng/mg
Cob (56 days)	2.2 ng/μg	2.5 ng/mg
Husk (56 days)	1.1 ng/μg	4.2 ng/mg
Silk	not detected[a]	
Stalk (24 days)	2.0 ng/μg	15.7 ng/mg
Stalk (77 days)	4.6 ± 0.4 ng/μg	11.2 ± 2.7 ng/mg
Immature tassel (49 days)	2.0	30.8 ng/mg
Pollen	not detected[b]	
Silage	not detected[b]	

[a] <0.05 ng/μg
[b] <0.08 ng/μg

2. Expression of the β-lactamase Gene

DNA sequence analysis of the β-lactamase gene cloned from the B16 line indicated that 568 base pairs of the 858 base pair β-lactamase gene are present (DNA sequence in Appendix 1). However, restriction enzyme mapping data and sequence analysis indicated that the 5′ region of the β-lactamase gene present in plasmid pDPG165 is intact in the B16 line. Therefore, the bacterial promoter region for the β-lactamase gene is present in B16, but the gene is truncated in the 3′region. It was determined (1) that the truncated β-lactamase gene present in the B16 line did not, when introduced into *E. coli*, produce active β-lactamase, and (2) that the truncated β-lactamase gene present in the B16 line does not produce any protein detectable on a Western blot.

a. Expression of the B16 β-lactamas Gene in E. coli

The *BamHI/NsiI* restriction enzyme digestion fragment comprising 2.5 Kb of the pDPG165 insert in the B16 line and about 1 Kb of flanking maize genomic DNA was cloned in a plasmid vector. This DNA sequence contains the β-lactamse gene and 5′ controlling regions that are present in the B16 line. The *BamHI/NsiI* DNA fragment was cloned into a plasmid vector that lacks a β-lactamse gene, but contains a gene encoding neomycin phosphotransferase II (*neo*) that confers kanamycin resistance on the bacterial host cell. It was determined that the truncated β-lactamase gene present in the B16 line does not confer resistance to 100 μg/ml ampicillin on *E. coli* cells containing the gene, whereas growth of *E. coli* cells containing an intact β-lactamase gene is unaffected by this concentration of ampicillin. Therefore, it was concluded that the truncated β-lactamase structural gene present in the B16 line is not functional.

Expression of the B16 truncated β-lactamase gene was further investigated by Western blot analysis. Anti-β-lactamase polyclonal antibody was obtained commercially (5′Prime → 3′Prime, Inc., Boulder, CO). β-lactamse antibody can detect as little as 100 ng of protein. Crude protein extracts were prepared from *E. coli* cells containing an intact β-lactamase gene, and therefore resistant to ampicillin, and *E. coli* cells containing the truncated β-lactamse gene derived from the B16 line, and not resistant to the antibiotic ampicillin. Protein was separated on polyacrylamide gels, blotted to nitrocellulose and probed with anti-β-lactamse antibody. β-lactamase protein was visualized in extracts prepared from *E. coli* cells containing an intact β-lactamse gene, but no β-lactmase protein was observed in extracts prepared from cells containing the truncated B16 β-lactamase gene. Therefore, the β-lactamase gene contained in the B16 line does not produce an immunologically detectable protein in *E. coli*.

b. Expression of the β-lactamase Gene in the B16 Line

The β-lactamase gene introduced into maize is under control of prokaryotic regulatory sequences for which there is no evidence that these elements can function in higher plants. Therefore, one would not expect the β-lactmase gene to be expressed in the B16 line. This result was confirmed by Northern and Western blot analysis of the B16 line. Northern blot analysis of the B16 line demonstrated that an mRNA species that hybridizes to the β-lactamse structural gene is present in *E. coli* cells containing the intact β-lactmase gene. However, as expected, there is no detectable mRNA produced in the B16 line. Furthermore, Western blot analysis of the B16 line demonstrated that the intact β-lactamase gene expresses an immunologically detectable protein in *E. coli*, but there is no immunologically detectable β-lactamse protein in the B16 line. The absence of β-lactamase mRNA and immunologically detectable β-lactamase protein in the B16 line, in combination with the inability of the truncated β-lactamase gene to confer resistance to ampicillin on *E. coli* cells, is strong evidence that the B16 line does not have the genetic capacity to produce a protein with β-lactamse activity, and as expected there is no β-lactamase produced in the B16 line.

VI. Agronomic Performance and Compositional Analysis of Glufosinate Resistant Line B16

The glufosinate resistant B16 line has been field tested by DEKALB since 1991 under APHIS permit and notifications listed in Table V1.1. The B16 line has been field tested at about 26 sites in the United States, as well as in 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 glufosinate resistance present in each one. Second, efforts have continued since 1990 to introgress the glufosinate resistance trait into a wide spectrum of DEKALB elite inbreds. Third, in 1994 finished glufosinate resistant hybrids were evaluated for agronomic performance and the effects of herbicide application on agronomic performance. Throughout this period qualitative observations on disease and pest resistance have been made. Appendix 2 contains termination reports of all field tests completed in the United States.

Table V1.1 APHIS Permits and Notifications

APHIS NUMBER	LOCATION
90-332-02*	Kihei, HI
90-332-04*	DeKalb, IL
91-317-01*	Kihei, HI
92-034-01*	DeKalb, IL
92-365-04*	Kihei, HI
93-014-02*	DeKalb, IL (3 sites); Mason City, IL; Illiopolis, IL; Thomasboro, IL; Windfall, IN Atlantic, IA; Dayton, IA, North Liberty, IA; Spencer, IA; Gorden City, KS; Mason, MI; Olivia, MN; Owatonna, MN; Glenvil, NE; Manheim, PA; Lima, OH; Brandon, SD; Dumas, TX; Madison, WI
94-081-02N	Olivia, MN; Owatonna, MN
94-081-04N	Leesburg, GA; Crescent City, IL; DeKalb, IL; Mason City, IL; Thomasboro, IL; Waterman, IL; Washington, IN; Windfall, IN; Atlantic, IA; Dayton, IA; North Liberty, IA; Spencer, IA; Mason, MI; Marshall, MO; Glenvil, NE; Mt. Olive, NC; Marion, OH; Manheim, PA; Brandon, SD, Union City, TN; Madison, WI
94-081-05N	Kihei, HI
94-088-01N	Sublette, KS
94-109-06N	West Lafayette, IN

* Termination reports filed with APHIS and included in Appendix 2. Termination reports have not been filed on the remaining sites as releases are not completed or followup monitoring for volunteers has not been completed.

A. Agronomic Performance

Qualitative observations were made on inbreds and hybrids as part of the breeding program of glufosinate resistant corn in the time period from 1990 through 1994. During this period no agronomic differences were observed in the glufosiante resistant lines other than the glufosinate resistance trait that was being introgressed into the inbred and hybrid lines (see termination reports, Appendix 2).

Seven DEKALB elite hybrids into which the glufosinate resistance trait from line B16 had been introgressed were evaluated for agronomic performance in 1994. Hybrids spanned relative maturities from 88 days to 112 days and were tested in the appropriate maturity zones at 12 field locations across the midwest. Hybrids were evaluated for yield, grain moisture, test weight, root and stalk lodging, dropped ears, barrenness, stay green, intactness, seedling vigor, plant height, ear height, time to pollen shed and silking, and green snap. For all traits each hybrid was compared to its glufosinate sensitive counterpart as well as evaluated following applications of 0.37 pounds glufosinate/acre and 1.48 pounds glufosinate/acre. The goal of this field trial was to determine whether the *bar* gene present in the B16 line had an effect on agronomic performance and to determine whether agronomic performance was effected by applications of glufosinate.

In general, the glufosinate resistant backcross converted versions of hybrids performed similarly to their unconverted counter parts. However, a number of small, but significant changes were observed. For example, one hybrid silked one-half day earlier whereas a second hybrid silked three-quarter's day later. Similarly, one hybrid produced grain with an increased test weight whereas a second hybrid produced grain with a decreased test weight. Two hybrids exhibited a statistically significant increase in yield, and three other hybrids exhibited an increase in seedling vigor. Agronomic performance was independent of herbicide application, suggesting that the application of glufosinate at concentrations up to 1.48 pounds active ingredient/acre had no effect on agronomic performance. Although stand counts were not taken, any differences in seedling germination would have been reflected in agronomic performance data. Agronomic performance testing will be repeated in 1995.

B. Disease and Pest Characteristics

Glufosinate resistant line B16 has been field tested in the United States since 1990, and in 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. Glufosinate resistant line B16 was visually inspected at the sites and time listed in Table VI.2. These represent 35 trials at sites in the continental United States in 14 states between 1991 and 1994. In addition the B16 line has been grown continuously in Hawaii (3-4 generations per year) since March, 1991. No changes relative to control plants have been observed in any of these tests with regards to disease or insect susceptiblity.

Table VI.2. Disease and Insect Pest Evaluation.

State	Location	Year	Differences in Susceptibility	
			Insect	Disease
Georgia	Leesburg	1994	None	None
Hawaii	Kihei	1991	None	None
		1992	None	None
		1993	None	None
		1994	None	None
Illinois	DeKalb	1991	None	None
		1992	None	None
		1993	None	None
		1994	None	None
	Mason City	1993	None	None
		1994	None	None
	Thomasboro	1993	None	None
		1994	None	None
Indiana	Washington	1994	None	None
	West Lafayette	1994	None	None
	Windfall	1993	None	None
		1994	None	None
Iowa	Atlantic	1993	None	None
		1994	None	None
	Dayton	1993	None	None
		1994	None	None
	North Liberty	1993	None	None
		1994	None	None
	Spencer	1993	None	None
		1994	None	None
Kansas	Garden City	1993	None	None
	Sublette	1994	None	None
Minnesota	Olivia	1994	None	None
	Owatonna	1994	None	None
Missouri	Marshall	1994	None	None
Nebraska	Glenvil	1993	None	None
		1994	None	None
Ohio	Lima	1993	None	None
	Marion	1994	None	None
Pennsylvania	Manheim	1993	None	None
		1994	None	None
South Dakota	Brandon	1994	None	None
Tennessee	Union City	1994	None	None
Wisconsin	Madison	1994	None	None

C. 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 alcohols, 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 primarilty localized in the embryo, i.e., about 83% of the fat or oil is found in the embryo. Table V1.3 gives the composition of corn grain, both with a range of values normally found in corn and average values.

Usually the grain or silage is used for animal feed. Compositional (proximate) analyses were performed on corn grain from line B16. Grain from glufosinate resistant hybrids was analyzed from seven hybrids grown in Hawaii. Control grain values represent averages of the same seven hybrids grown throughout the midwest during 1994. There was a statistically significant increase in the level of protein and corresponding decrease in the level of starch in the B16 grain that was analyzed (Table VI.3). Despite these differences, the composition of B16 grain falls within the range observed for corn hybrids. Differences may be attributed to environmental effects on grain composition and, therefore, DEKALB will conduct additional compositional analyses on grain produced from side-by-side grown B16 and corresponding unconverted hybrids during the summer of 1995. No compositional analyses were undertaken on silage as there was no detectable PAT protein in silage and, therefore, no expected impact of the presence of the *bar* gene on the composition of silage.

Table VI.3. Composition Analysis of the B16 Line

Component	B16 Hybrid	Counterpart Unconverted Hybrid	DEKALB Hybids (Range/Average)[a]
Oil[b]	4.54	4.18	2.0-5.9%/4.1%
Protein[c]	12.18*	9.26	6.8-13.4%/9.2%
Starch[d]	72.8*	74.8	64-79.3%/74.4%

*Difference is significant at the 5% level of significance between the transformation events and their nontransgenic counterparts based on a Student's t-test.
[a]Ranges and averages for DEKALB hybrids are derived from analysis of commercial DEKALB hybrids grown throughout the midwest in 1993 and 1994.
[b]Oil content was determined by near-infrared reflectance spectrometry calibrated to lab analysis by AAAC (1980) method 30-20.
[c]Protein content was determined by near-infrared reflectance spectrometry calibrated to lab analysis by Kjeldahl method (Koops, J. et al., 1975) Conversion to protein is 6.25 X N.
[d]Starch content was determined by near-infrared reflectance spectrometry calibrated by starch analysis in which starch is digested (α-amylase, amyloglucosidase) and the resulting glucose is measured by a hexose kinase/6-phophoglucose dehydrogenase assay (Karkalas, 1985).

VII. Environmental Consequences of Introduction of Line B16

A. The Herbicide Glufosinate, Herbicide Usage in Corn and the Impact of Glufosinate Resistant Corn on Current Practices

1. The Herbicide Glufosinate

Several members of the genus *Streptomyces*, including *S. hygroscopicus* and *S. viridochromogenes*, are capable of producing the tripeptide antibiotic phosphinothricyl-alanyl-alanine. The active moiety of the tripeptide is the glutamate analogue phosphinothricin, or glufosinate, which is released from the tripeptide by intracellular peptidase activity and has been demonstrated to be an inhibitor of the enzyme glutamine synthase in both prokaryotes and eukaryotes. In plants, glutamine synthase is a key enzyme in nitrogen metabolism and is responsible for assimilation of ammonia into organic compounds. Glutamine synthase catalyzes the addition of ammonia to α-ketoglutarate to produce the amino acid glutamic acid. In the absence of glutamine synthase activity, e.g., when glutamine synthase activity is inhibited by glufosinate, ammonia levels increase and cell death follows. De Block et al. (1987) reported that ammonia levels increase 40-fold within 8 hours of application of glufosinate. Significantly, ammonia levels did not increase over a 24 hour period in glufosinate resistant plants.

Glufosinate is a broad spectrum non-selective contact herbicide with no systemic activity in corn. The compound is rapidly metabolized by microbial activity in the soil and hence there is minimal risk of persistence and groundwater contamination. The major advantage of glufosinate is rapid burn down of existing vegetation and broad spectrum activity. At the expected field application rates in corn, glufosinate is expected to be more effective on broad leaf species than grasses. Currently glufosinate is not registered for use in corn. It has been registered for use on turf (under the tradename Finale®) and for use on apples, grapes, and tree nuts (under the trade name Rely®). Glufosinate is registered for use on plantation crops, tree nuts, and vines, and for non-agricultural weed control (under tradenames including Basta® and Ignite®) outside the United States.

2. Current Usage of Herbicides on Corn

Corn has been grown on 60 million to 83 million acres per year in the period from 1982 to 1993. In 1993, fifteen states had corn acreages in excess of one million acres, and 74% of the crop was grown in Iowa, Illinois, Nebraska, Minnesota, and Indiana. Herbicides were applied to about 97% of the corn acreage in the United States, and over 98% of the corn acreage in Iowa,

Illinois, Minnesota, and Indiana had herbicide applications (Agricultural Chemical Usage, 1994). Furthermore, an average of 2.1 active ingredients were applied per acre in 1992.

A diverse group of weed species necessitates a range of weed control methods in corn. Broad leaf weeds such as velvetleaf, pigweed, wild sunflowers, ragweeds, and smartweeds are of concern in corn. Furthermore, grass weeds such as johnson grass, shattercane, fall panicum, foxtails, quackgrass, wild proso millet and wooly cupgrass are common in corn. Perennial weeds are an additional problem as they are able to propagate by seed and/or underground plants parts, and may necessitate multiple herbicide applications. The wide array of weed species that are found in corn field requires the use of multiple type of herbicides and multiple applications in order to achieve weed control. Therefore, herbicide application regimes vary depending on the weed spectrum and local agronomic practices. Table VII.1 summarizes herbicide treatment of corn acreage in 1993.

Table VII.1 Herbicide Applications to Corn

Percent of Acres Treated with Major Corn Herbicides		
Herbicide Name	**Major Corn Growing States (Including Minn.)**	**Minnesota**
Atrazine	69	37
Metolachlor	32	24
Alachlor	24	23
Dicamba	21	48
Cyanazine	20	16
2, 4-D	12	13
Bromoxynil	8	14
Nicosulfuron	6	19

Source: Agricultural Chemical Usage, March 1994, NASS and ERS, USDA.

A single application of herbicides near the time of planting is most common in corn. Usually this application comprises one of the triazine herbicides (atrazine, cyanazine, simazine) to control broadleaf weeds and an acetanilide herbicide (metolachlor, alachlor) to control annual grasses. Control of broadleaf weeds and problem grasses with postemergent herbicides such as dicamba, bromoxynil, bentazon, nicosulfuron and primisulfuron, occurred on about half of the corn acreage in

1993. Choice of herbicide is consistent in all but the north central states (e.g., Minnesota and South Dakota). Atrazine was used on about 69% of the corn acreage in 1993. The most common tank mix was atrazine and metolachlor for broad spectrum weed control. Herbicide usage in the north central states, however, differs in that there is reduced usage of atrazine due to carryover to small grains and soybeans in the high pH, low rainfall soils of the region. Furthermore, because the growing season is shorter in the north central region, postemergent herbicdes are preferred in that they do not delay planting operations. For example, in 1993, the most common herbicide used on corn in Minnesota was the postemergent herbicide dicamba (all data from Agricultural Chemical Usage, 1994).

3. Possible Effects of Glufosinate Resistant Corn on Current Agricultural Practices

The application of glufosinate to glufosinate resistant corn will not effect the normal growth of the crop or negatively impact its agronomic performance. The availability of glufosinate for weed control in corn will give the farmer increased flexibility in dealing with weed problems. The growing of glufosinate resistant corn hybrids will offer the farmer 1) the use of a new herbicide which offers broad spectrum control of annual and perennial, broad leaf and grass weeds; 2) less dependence on pre-plant herbicide applications; 3) increased flexibility in applying herbicides on an as needed basis; 4) a new herbicidal mode of action which will decrease the likelihood of development of herbicide resistant weeds; and 5) a herbicide for use in no-till systems which conserve fuel and reduce soil erosion. Because of the advantages offered, post-emergent herbicides are being applied to increasing acreages of corn every year, e.g., about 15 million acres of corn, 20% of the total corn acreage, receive only post-emergent herbicide applications. Glufosinate resistant corn will provide the farmer with an alternative weed control method. Currently on the average 2.1 herbicides are applied to corn during the growing season. It is expected that the use of glufosinate for weed control will reduce the number of kinds of herbicides applied as well as the number of required applications.

B. The Likelihood of Appearance of Glufosinate Resistant Weeds

The application of a herbicide to the same area over an extended period of time will change the characteristic weed population of that area. For example, applications of 2,4-D and atrazine in corn in the midwest has resulted in the replacement of broad leaf weeds with grass species as the predominant weeds in corn. Furthermore, development of weed and crop varieties with resistance to particular herbicides has been documented. Triazine resistant biotypes of common lambsquarter, smooth pigweed, and Powell amaranth have been identified. The

development of resistant types is not limited to weed species, but has been documented in crops as well. For example, the resistance of corn to triazine herbicides is controlled by a single recessive gene and, therefore, there are triazine sensitive and resistant lines of corn. The ability of plants to become herbicide resistant through spontaneous mutations has been used to produce imidazolinone resistant corn lines (Anderson and Georgeson, 1989) and sethoxydim resistant corn lines (Parker et al, 1990).

Glufosinate is a mixed-competetive inhibitor of the enzyme glutamine synthase (GS; Leason et al., 1982) which facilitates the incorporation of inorganic ammonia into organic compounds in the plant throught catalysis of the enzymatic addition of ammonia to glutamate to form glutamine. In the absence of GS activity ammonia accumulates to toxic levels in the cell.

A number of GS isozymes can be identified in plant cells. One isozyme is localized in the chloroplast (GS_2) and the remaining isozymes (GS_1 species) are cytosolic. In both rice (Hirel et al, 1982) and wheat (Tobin et al, 1985) chloroplast isozymes of GS appear to be the major forms active in photosynthetic tissue. The glutamine synthase gene family in *Arabidopsis thaliana* has been investigated in detail and it appears that there are at least three cytoplasmic GS isozymes and one chloroplast form (Peterman and Goodman, 1991) Furthermore, in *Arabidopsis* one GS isozyme is leaf specific (the putative chloroplastic isozyme), two isozyme species are root and germinated seed specific, and a third isozyme species is primarily root specific. The putative chloroplastic GS isozyme is light-inducible in *Arabidopsis* as it is in rice (Hirel et al., 1982). In corn six GS genes have been identified (Maize Genetics Cooperation Newsletter, 1994). The corn glutamine synthase 7 isozyme is localized in the chloroplast. The remaining isozyme species show similar tissues specificities to those isozymes identified in *Arabidopsis*, i.e., one isozyme is young seedling specific, two isozymes are root and leaf specific, and two isozymes are root specific.

The development of glufosinate resistant weeds in unlikely for several reasons. Glufosinate has no soil residual activity and is rapidly broken down by microorganisms. Therefore there is no long term exposure of weed populations to the herbicide. In addition, glufosinate presents a new herbicidal mode of action and, therefore, will not increase the selective pressure already applied to weed populations by herbicides with a particular mode of action, e.g., acetolactate synthase inhibitors such as the imidazolinones and sulfonylureas.

There are three basic mechanisms by which glufosinate resistant plants may develop. The target enzyme, glutamine synthase, could mutate and produce a herbicide insensitive variant of the enzyme. Alternatively, the plant could overproduce glutamine synthase and

thereby overcome enzyme inhibition. This method of resistance is unlikely to produce plants with high levels of resistance. The most efficient way of conferring glufosinate resistance on a plant is to enable the plant to inactivate glufosinate so that it does not inhibit glutamine synthase activity. It is this mechanism of glufosinate resistance that is conferred by the *bar* gene.

The complex nature of regulation of GS isozymes including tissue specific and developmental stage specific expression, and separate genes coding for these isozymes, make it highly unlikely that a single mutation in glutamine synthase will render a plant resistant to glufosinate. Whereas a glufosinate resistant chloroplastic form of GS may confer a level of glufosinate resistance on the plant, it probably would not confer high level resistance or resistance on all vegetative parts of the plant. It is likely that a series of gene mutations in chloroplast and cytoplasmic GS genes would be required to make a plant resistant to glufosinate. Furthermore, GS has a multimeric subunit structure and enzyme complexes comprising glufosinate resistant and sensitive subunits may be inhibited by the herbicide. Homozygous mutants would be necessary to confer herbicide resistance through GS enzymes comprised completely of glufosinate resistant subunits. These mutations would therefore be recessive and would not be directly selected for under herbicide pressure. There is, therefore, a low probability that glufosinate resistant weeds will evolve by mutation.

Donn et al. (1984) reported selection of alfalfa suspension cultures resistant to glufosinate. Glufosinate resistance was achieved through a three to seven-fold increase in GS activity in resistant cells as a consequence of amplication of one of the GS genes. No plants were regenerated from this cell line, but the amplified GS gene was isolated and introduced into *Nicotiana tabacum* (Eckes et al., 1989). In alfalfa GS expressing tobacco plants GS comprised up to 5% of the total protein in the cells and plants were 20-fold more resistant to glufosinate than normal tobacco plants. However, it is not clear whether converting more glutamate to glutamine will have undesirable effects on nitrogen metabolism in the plant or confer sufficient levels of herbicide resistance to allow a plant to be competetive in the absence of herbicide pressure. Indeed, Deak et al. (1988) suggested that the lack of plant regeneration of glufosinate resistant alfalfa cell cultures with an amplified GS gene may be due to significant changes in nitrogen metabolism in the cells. It is therefore unlikely that weed resistance to glufosinate will evolve through gene amplification.

The third mechanism for producing glufosinate resistant plants is through inactivation of the herbicide in the plant. This is the mechanism of resistance in the corn line B16 and it is unlikely that such a mode of herbicide resistance will appear in weed

species unless the gene is introduced from a plant into which a gene encoding a phosphinothricin acetyltransferase gene has been introduced. There are no sexually compatible species of corn in the United States and therefore it is unlikely that the *bar* gene present in line B16 will be transferred to other species. Furthermore, since the *bar* gene specifically confers resistance to glufosinate on the corn plant, plants derived from the B16 line will not be resistant to other classes of herbicides.

It is, therefore, unlikely that weeds resistant to glufosinate or other herbicides will evolve as a result of the introduction of the glufosinate resistant corn line B16.

C. Weediness Potential of the Line B16

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 single 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.

Keeler (1985) summarized the weediness potential of genetically engineered crops. Keeler suggests that there is no reason *per se* why geneticlly engineered crops would be more likely to become weeds or contribute to the weediness of other plants than new varieties of crops produced by conventional methdos. The impetus for weediness ultimately comes from selection in the field and the ability of the plant species to adapt to that selection.

In conclusion, glufosinate resistant corn is unlikely to become a weed or contribute to the weediness of other species.

D. Potential for Gene Transfer to Other Organisms

There are no plant species with which corn hybridizes present in the United States. Outcrossing to related species was addressed in section II.F. There is no potential for outcrossing of glufosinate resistant corn to other plant species in the United States.

Hybridization between cultivars of corn can freely occur unless measures are taken to isolate a cultivar. In production of hybrid seed for commercial sale, the glufosinate resistant inbred parent will be grown in reproductive isolation from all inbreds except the second parent of the hybrid in order to meet seed certification requirements. Therefore under these conditions it is highly unlikely that a glufosinate resistant inbred would unintentionally hybridize with a second corn variety. It is anticipated that a glufosnate resistant inbred may be used as a breeding start for development of novel improved inbreds. Under these conditions the glufosinate resistant inbred will be part of controlled pollination experiments and therefore unlikely to freely cross hybridize with other varieties of corn. Controlled pollinations are the basis of breeding efforts in any crop species. 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 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 glufosinate resistance trait will 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.

It is highly unlikely that transgenes will be transferred from glufosinate resistant corn to other organisms that are not sexually compatible with corn if glufosinate resistant 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 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 existense of any mechanism, other than sexual crossing," for the transfer of a transgene from a plant to a sexually incompatible organism. In the unlikely event that the *bar* gene were transferred to a microorganism, it would present the organism with no selective advantage in the absence of glufosinate. Furthermore, the *bar* gene was isolated from the common soil microorganism *Streptomyces hygroscopicus* and, therefore, is already present in soil microorganisms. In the event that the β-lactamase gene present in the B16 line were transferrred to microorganisms, this gene would confer no selective advantantage on the microorganisms since it is a truncated gene and does not encode a functional β-lactmase gene. Furthermore, genes encoding β-lactamase are commonly found in microorganisms. The DNA insertion in the B16 line does not contain a prokaryotic origin of replication. It is, therefore, unlikely that a segment of DNA could be transferred from a plant of the B16 line to a microorganism and be maintained therein as an independently replicating DNA element.

In conclusion, it is highly unlikely that the transgenes introduced into the B16 line could be transferred to sexually incompatible species of plants.

E. Effects of Glufosinate Resistant Corn on Non-Target Organisms

The B16 line of glufosinate resistant corn has been tested at about 24 locations in 14 states as well as Argentina and Canada from 1991 throug 1994. During that period no effects on populations of birds or other wildlife or changes in insect or disease susceptibility have been observed (see Termination reports in Appendix 2). Furthermore, the agronomic performance of corn hybrids derived from the B16 line is comparable to glufosinate sensitive corn hybrids and, therefore, no changes in effects on non-target organisms were expected. No effects on non-target organisms were expected, because the *bar* gene was derived from a common soil microorganism and has no homology to genes of known toxins or allergens (see section VI.F).

F. Corn Based Products and Human/Animal Exposure

The PAT protein encoded by the *bar* gene does not have any characteristics of an allergen or a toxin. The amino acid sequence of the PAT protein has no glycosylation motifs. The gene only shows homology to other genes encoding PAT proteins, and the PAT protein shows homology only to other PAT proteins. There is no homology to any other gene or protein sequences in

GenBank, including known toxins and allergens. Furthermore, the PAT protein encoded by the *bar* gene is heat and pH labile and has no glycosylation motifs. The PAT enzyme activity is highly specific for acetylation of phosphinothricin and shows no activity with glutamate, of which phosphinothricin is an analogue, or other glutamate analogues (Thompson et al., 1987). Furthermore, DEKALB Genetics has demonstrated that PAT has no activity on the mixture of amino acids present in a casein hydrolysate mixture. Amino acid acetyltransferase activities are common in living organisms and therefore, PAT does not represent a novel type of enzyme activity. It is, therefore, unlikely that the PAT protein present in the B16 line will expose organisms to a new toxin or allergen.

The uses of corn were discussed above in section VI.C. The primary expsosure of animals and humans to glufosinate resistant corn will be through oral ingestion. In the B16 line, there is no evidence for expression of the β-lactmase gene and the gene itself is truncated and does not encode a functional protein. Therefore, there is no anticipated exposure of animals and humans to β-lactamase in the B16 line. As proteins are water soluble, it is not anticipated that the PAT protein will be present in corn oil.

As discussed in section VI.C, over 80% of the corn crop produced in the United States is used for animal feed. Animal feed is for cattle (40%), swine (35%) and poultry (25%). Digestive systems in all of these animals comprise at least an acid stomach (e.g., pH 1) with active peptidases such as pepsin, and an intestine at neutral pH with active peptidases such as trypsin and chymotrypsin. In addition, cattle use microbial fermentation in the rumen to pre-digest plant material prior to digestion in the stomach and intestine. The rumen comprises an additional neutral pH digestion step and includes microbial protease activity.

The stability of PAT protein encoded by the *bar* gene was investigated in simulated gastric and intestinal fluids. *In vitro* digestion assays were performed using simulated gastric and intestinal fluids (The United States Pharmacopeia, 1990) and protein extracts from B16 leaves. The presence of PAT protein was assayed by Western blot. Fifty to one hundred fifty ng of PAT was present in about 50 μg plant protein when incubated in simulated digestive fluid. The PAT Western with chemiluminescent detection is sensitive to below 10 ng per lane. No trace of PAT protein was detectable after 1 minute in simulated gastric fluid. PAT protein was stable in neutralized simulated gastric fluid, suggesting that protein degradation was a result of pepsin activity. Stability of PAT protein in simulated intestinal fluid was also investigated. No PAT protein was detected following 10 seconds incubation in simulated intestinal fluid. Therefore, even if PAT protein survived in a neutralized gastric

environment, it would be rapidly degraded in the intestine. In conclusion, PAT protein will not survive in the digestive system of animals and, therefore, animal exposure to the protein will be minimal.

The remaining corn grain that is not used directly as feed is processed by wet and dry milling to produce a variety of products. The wet and dry milling processes are described in Watson (1987), Alexander (1987) and May (1987). In the wet milling process water soluble PAT protein will be extracted into the steep in the initial 48-52°C incubation for up to 30 hours. DEKALB Genetics has shown that PAT enzyme activity is not detectable following a 4 hour incubation at 50°C. Furthermore, no PAT enzyme activity was detected following a 24 hour incubation at ambient temperature (25°C). There was a greater than 10-fold reduction in the amount of PAT protein as detected by Western blot analysis following incubation for 24 hours at 50°C. Therefore, no PAT protein will be present in the corn steep that is concentrated to produce corn steep liquor for use in fermentation or in combination with fiber to produce corn gluten feed for animals.

Dry milled grain is used for production of animal feed as well as in fermentation, and preparation of various food products. PAT protein present in dry milled animal feed will be degraded by the digestive system as described above. Dry milled corn products for human consumption are usually heat treated at one step in the process and therefore PAT protein will be degraded. For example, in preparation of breakfast cereals dry milled corn is treated with steam to gelatinize starch. In recent years, dry milled corn has been used in corn-soy-milk which contains heat treated gelatinized corn meal. Dry milled corn is also extensively used for industrial purposes such as charcoal briquette binders, adhesive for gypsum board, paperboard, oil well drilling mud thickeners, and others in which human or animal exposure would be minimal and processing would be expected to degrade any PAT protein present.

In conclusion, the PAT protein has no homology to known allergens or toxins, no glycosylation motifs and is both heat and pH labile. PAT is rapidly degraded in both simulated gastric and intestinal fluids. Furthermore, processing of corn for the production of food products for humans or animals will most likely destroy the PAT protein. The PAT protein is produced at low levels in the B16 line. The highest level of expression of the PAT protein occurs in leaf and stalk in which the PAT protein represents up to 0.46% of the total soluble protein. In mature grain the concentration of PAT protein is about 100-fold lower than in leaf or stalk, and there is no detectable presence of the PAT protein in silage. The PAT protein will therefore be a minor constituent of the animal or human diet and based on the properties of the protein, no adverse effects are expected.

VIII. Statement of Grounds Unfavorable

No unfavorable information and data has been demonstrated for the glufosinate resistant corn line B16.

IX. Literature Cited

Association of Official Analytical Chemists. 1980. Official Methods of Analysis 13th edition. Secs. 7.055-7.056, p. 132.

Agricultural Chemical Usage, March 1994, NASS and ERS, USDA.

Alexander, R.J., 1987. Corn Dry Milling: Processes, products and applications. In Watson, S.A. and Ramstad, P.E., Corn Chemistry and Technology.

Anderson, P.C. and Georgeson, M. 1989. Herbicide-tolerant mutants of corn. Genome 31: 994-999.

Baker, H.G., 1974. The evolution of weeds. Ann. Rev. Ecol. Syst. 5: 1-24.

Baulcombe, D.C., Saunders, G.R., Bevan, M.W., Mayo, M.A., and Harrison, B.D., 1986. Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature 321: 446-449.

Bayer, E., Gugel, K.H., Hagele, K., Hagenmaier, H., Jessipow, S., Kõnig, W.A., Zähner, H. 1972. Stofwechproducte von Mikroorganismen. Phosphinothricin und Phosphinothricyl-alanyl-alanin. Helvetica Chimica Acta 55: 224-239.

Bevan, M.W., Mason, S.E., and Goelet, P. 1985. Expression of tobacco mosaic virus coat protein by cauliflower mosaic virus promoter in plants transformed by *Agrobacterium*. The EMBO J. 4: 1921-1926.

Burr, B, Burr, F.A. and Matz, E. 1994. Mapping genes with recombinant inbreds. In Freeling, M. and Walbot, V., eds. The Maize Handbook.

Deak, M., Donn, G., Feher, A. and Dudits, D. 1988. Dominant expression of a gene amplification-related herbicide resistance in medicago cell hybrids. Plant Cell Reports 7: 158-161.

De Block, M., Botterman, J., Vandewiele, M., Dockx, J., Thoen, C., Gosselé, V., Rao Movva, N., Thompson, C., Van Montagu, M. and Leemans, J. 1987. Engineering herbicide resistance in plants by expression of a detoxifying enzyme. The EMBO J. 6: 2513-2518.

Dhaese, P., De Greve, H., Gielen, J., Seurinck, J., Van Montagus, M., and Schell J., 1983. Identification of sequences involved in polyadenylation of higher lant nuclear transcripts using *Agrobacterium* T-DNA genes as models. The EMBO J. 2: 419-426.

Donn, G., Tischer, E., Smith, J.A. and Goodman, H. 1984.

Herbicide resistant alfalfa cells: an example of gene amplification in plants. J. of Molec. and Applied Genet. 2: 621-635).

Eckes, P., Schmitt, P., Daub, W., and Wengenmayer, F. 1989. Overproduction of alfalfa glutamine synthase in transgenic tobacco plants. Mol. Gen. Genet. 271: 263-268.

Franck, A., Guilley, H., Jonard, G., Richards, K., and Hirth, L. 1980. Nucleotide sequence of Cauliflower Mosaic Virus DNA. Cell 21: 285-294.

Galinat, W.C., 1983. The origin of corn as shown by key morphological traits of its ancestor teosinte. Maydica 28: 121-138.

Gordon-Kamm, W.J., Spencer, T.M., Mangano, M.L., Adams, T.R., Daines, R.J., Start, W., O'Brien, J.V., Chambers, S.A., Adams Jr., W.R., Willets, N.G., Rice, T.B., Mackey, C.J., Krueger, R.W., Kausch, A.P., Lemaux, P.G. 1990. Transformation of maize cells and regeneration of fertile transgenic plants. The Plant Cell 2:603-618.

Guilley, H., Dudley, R.K., Jonard, G., Balazs, E. and Richards, K.E., 1982. Transcription of Cauliflower Mosaic Virus DNA: detection of promoter sequences, and characterization of transcripts. Cell 30: 763-773.

Hirel, B., Vidal, J., and Gadal, P. 1982. Evidence for a cytosolic-dependent light induction of chloroplastic glutamine synthase during greening of etiolated rice leaves. Planta 155: 17-23.

Kamo, K.K. and Hodges, T.K. 1986. Establishment and characterization of long-term embryogenic maize callus and cell suspension cultures. Plant Science 45: 111-117.

Karkalas, J. 1985. An improved enzymatic method for the determination of native and modified starch. J. Sci. Food Agric. 36: 1019-1027.

Keeler K.H., 1985. Implications of weed genetics and ecology for the deliberate release of genetically engineered crop plants. Recombinant DNA Technical Bulletin 4: 165-172.

Keeler, K.H., 1989. Can genetically engineered crops become weeds? Bio/Technology 7: 1134-1139.

Koops, J., Klomp, H. and Elgersma, R.H.C. 1975. Rapid determination of nitrogen in milk and dairy products by colorimetric estimation of ammonia following an accelerated digestion procedure. Neth. Milk Dairy J. 29: 169-180.

Laughnan, J.R. and Gabay-Laughnan, S. 1994. The placement of genes using *waxy*-marked reciprocal translocations. In Freeling, M. and Walbot, V., eds. The Maize Handbook.

Leason, M., Cunliffe, D., Parkin, D., Lea, P.J., and Miflin, B. 1982. Inhibition of pea leaf glutamine synthase by methionine sulfoximine, phosphinothricin and other glutamate analogues. Phytochemistry 21: 855-857.

Maize Genetics Cooperation Newsletter 68: 158-208, 1994.

May, J.B. 1987. Wet Milling: Process and Products. In Watson, S.A. and Ramstad, P.E., Corn Chemistry and Technology.

Odell, J.T., Nagy, and Chua, N.-H. 1985. Identification of DNA sequences required for activity of the Cauliflower Mosaic Virus 35S promoter. Nature 313: 810-812.

Parker, W.B., Marshall, L.C., Burton, J.D., Somers, D.a., Wyse, D.L., Gronwald, J.W. and Gengenbach, B.G. 1990. Dominant mutations causing alterations in acetyl-coenzyme A carboxylase confer tolerance to cyclohexanedione and aryloxyphenoxypropionate herbicides in maize. Proc. Natl. Acad. Sci. 87: 7175-7179.

Peterman, T.K. and Goodman, H.M. 1991. The glutamine synthase gene family of *Arabidopsis thaliana*: light-regulation and differential expression in leaves, roots and seeds Mol. Gen. Genet. 230: 145-154.

Strauch, E., Wohlleben, W. and Pühler, 1988. Cloning of a phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its expression in *Streptomyces lividans* and *Escherichia coli*. Gene 63: 65-74.

Sutcliffe, J.G., 1978. Nucleotide sequence of the ampicillin resistance gene of *Escherichia coli* plasmid pBR322. Proc. Natl. Acad. Sci. USA 75: 3737-3741.

Tachibana K., Watanabe, T., Sekizawa, Y., Takematsu, T., 1986. Accumulation of ammonia in plants treated with bialaphos. J. Pesticide Sci. 11: 33-37.

The United States Pharmacopeia, Vol. XXII, p. 1788-1789. United States Pharmacopeial Convention, Inc. Rockville, MD, 1990.

Thompson, C.J., Movva, N.R., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M., and Boterman, J. 1987. Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. The EMBO J. 6: 2519-2523.

Tobin, A., Ridley, S.M., and Stewart, G.R. 1985. Changes in the

activities of chloroplast and cytosolic isoenzymes of glutamine synthase during normal leaf growth and plastid development in wheat. Planta 163: 544-548.

USDA-APHIS, 1991. Environmental Assessment and Finding of No Significant Impact. Permit Number 90-332-02.

USDA-APHIS, 1992. Interpretive Ruling on Calgene, Inc. Petition for Determination of Regulatory Status of FLAVR SAVR™ 57 FR 47608-47616.

Watson, S.A. 1987. Structure and Composition. In Watson, S.A. and Ramstad, P.E., eds. Corn Chemistry and Technology. American Association of Cereal Chemists, Inc.

Watson, S.A., 1987. Corn Marketing, Processing and Utilization in Sprague, G.F. and Dudley, J.W. eds. Corn and Corn Improvement.

White, J., Chang, S.-Y. P., Bibb, M.J. and Bibb, M.J. 1990. A cassette containing the *bar* gene of *Streptomyces hygroscopicus*; a selectable marker for plant transformation. Nucleic Acids Research 18: 1062.

Wohlleben, W., Arnold, W., Broer, I., Hillemann, D., Strauch, E. and Pühler, 1988. Nucleotide sequence of the phosphinothricin N-acetyltransferase gene from *Streptomyces viridochromogenes* Tü494 and its exppression in *Nicotiana tabacum*. Gene 70: 25-37.

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

95-145-01P

DEKALB Genetics Corporation

Petition for Determination of
Non Regulated Status:

Glufosinate Resistant Corn Line B16

#DG 95-128P

Appendices 1 and 2

Appendix 1A

β-Lactamase-Corn Genomic DNA Junction

The end of the B16 insertion 5' to *bar* was sequenced using a 21 base pair oligonucleotide primer homologous to the β-lactamase gene. The sequence of this primer corresponds to positions 590-610 of the sequence designated the complement of SYNPUC19 (sequence of plasmid pUC19 which contains the β-lactamase gene). This primer was used to generate sequence from a plasmid designated pDPG637. This plasmid contains a portion of the B16 insertion and genomic DNA that was cloned as an approximately 3 kb *BamHI/NsiI* fragment that hybridized to 35S (see Figure V.7 (a)). This fragment originally cloned into pBSSK(-) and designated pDPG636 and was subsequently cloned into a vector containing a *neo* gene and lacking a β-lactamase gene (pDPG637) to facilitate sequencing the cloned B16 copy of the β-lactamase gene. Sequence analysis demonstrated that the β-lactamase gene present in B16 was intact up to position 768 of the SYNPUC19 sequence (position 161 of the sequence generated from pDPG637) which corresponds to base pair 568 of the 858 base pairs of β-lactamase coding sequence. β-lactamase coding sequence initiates at position 201 in the SYNPUC19 sequence and ends at position 1058. Sequence generated beyond position 768 of SYNPUC19 was not homologous to the β-lactamase gene and was presumably maize genomic DNA sequence. This sequence analysis indicated that 290 base pairs of β-lactamase coding sequence were not present in B16. The β-lactamase DNA sequence found to be present in the clone of the B16 insert includes the *Asp700* (SYNPUC19 position 384-394) and *ScaI* (SYNPUC position 505-510) restriction sites that were found to be present by restriction mapping and Southern blot hybridization (section V.B.2.d). The β-lactamase sequence in B16 terminates before the *BsaI* site that is present in an intact β-lactamase gene (SYNPUC19 position 916-926). However, a *BsaI* site was found to be present in the maize genomic DNA region of the sequence generated from pDPG637 at position 417-427, accounting for the *BsaI* site determined to be present by restriction mapping (section V.B.2.d).

APPENDIX 1A

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Appendix 1B

Tr7-35S Promoter Junction

The Tr7-35S junction in the B16 insertion (see Figure V.7(a)) that resulted from recombination of two fragments of pDPG165 was sequenced to determine how much Tr7 and 35S sequence was present. Sequencing was performed using pDPG636 which contains the cloned 3 kb *BamHI/NsiI* B16 fragment containing the Tr7-35S junction (see Figure V.7(a)). An 18 base pair oligonucleotide primer corresponding to positions 390-407 of the sequence designated the complement of Tr7 was used to generate sequence from pDPG636. Sequence homologous to Tr7 was generated up to position 503 of the complement of Tr7. This position corresponded to position 98 of sequence generated from pDPG636. The Tr7 sequence generated from pDPG636 includes up to about 60 base pairs 3' to the *Asp700* site which is present at position 433-442 in the complement of Tr7. At position 99 of the pDPG636 sequence, homology to the 35S promoter initiated, corresponding to position 220 of the 35S sequence. The 35S sequence generated from pDPG636 includes the *ScaI* (35S sequence position 318-323) and *NcoI* (35S sequence position 418-423) restriction sites that were found to be present by restriction mapping and Southern blot hybridization (section V.B.2.a)

APPENDIX 1B

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Appendix 1C

Tr7-Corn Genomic DNA Junction

The Tr7-genomic DNA junction of the B16 insertion was sequenced to determine how much Tr7 sequence 3' to the *bar* gene was present. Sequencing was performed using a plasmid designated pDPG651 which is pBSSK(-) and containing *bar*, Tr7, and genomic DNA that were cloned from B16 as an approximately 2 kb *BamHI/HindIII* fragment (see Figure V.7(a)). An 18 base pair oligonucleotide primer corresponding to positions 173-190 of Tr7 sequence was used to generate sequence from pDPG651. Sequence homologous to Tr7 was generated up to position 559 of the Tr7 sequence. Sequence generated beyond that position was not homologous to Tr7 and was presumably genomic DNA. Position 559 of the Tr7 sequence corresponds to one base pair upstream of the *EcoRI* site (position 560-565) present in pDPG165. This indicates that the copy of Tr7 3' to *bar* in the B16 insertion is almost complete, ending just before and lacking the *EcoRI* site. This is in agreement with restriction mapping described in section V.B.2.c.

APPENDIX 1C

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Appendix 1D

Published DNA Sequences Contained in pDPG165

1. The plasmid backbone of pDPG165, including the β-lactamase gene was derived from pUC19. The DNA sequence of pUC19 is contained in:

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

2. The DNA sequence of Cauliflower Mosaic Virus including the 35S promoter is contained in:

   Franck, A., Guilley, H., Jonard, G., Richards, K., and Hirth, L. 1980. Nucleotide sequence of Cauliflower Mosaic Virus DNA. Cell 21: 285-294.

   The 35S promoter in pDPG165 is identical to the 35S promoter contained in the plasmid vector pROK1 described in:

   Baulcombe, D.C., Saunders, G.R., Bevan, M.W., Mayo, M.A., and Harrison, B.D., 1986. Expression of biologically active viral satellite RNA from the nuclear genome of transformed plants. Nature 321: 446-449.

   Construction of the 35S promoter contained in pROK1 is described in:

   Bevan, M.W., Mason, S.E., and Goelet, P. 1985. Expression of tobacco mosaic virus coat protein by cauliflower mosaic virus promoter in plants transformed by *Agrobacterium*. The EMBO J. 4: 1921-1926.

3. The DNA sequence of the *bar* gene is given in:

   White, J., Chang, S.-Y. P., Bibb, M.J. and Bibb, M.J. 1990. A cassette containing the *bar* gene of *Streptomyces hygroscopicus*; a selectable marker for plant transformation. Nucleic Acids Research 18: 1062.

4. The DNA sequence of the Tr7 3' region is given in:

   Dhaese, P., De Greve, H., Gielen, J., Seurinck, J., Van Montagus, M., and Schell J., 1983. Identification of sequences involved in polyadenylation of higher lant nuclear transcripts using *Agrobacterium* T-DNA genes as models. The EMBO J. 2: 419-426.

Appendix 2

TERMINATION REPORTS

TERMINATION REPORT FOR: Field Release Permit #90-332-02

Periods of release - March 14, 1991 through June 6, 1991
- April 3, 1991 through July 2, 1991
- June 13, 1991 through August 30, 1991
- July 11, 1991 through October 9, 1991
- September 6, 1991 through November 22, 1991
- November 20, 1991 through March 19, 1992
- December 4, 1991 through April 2, 1992

Trait - Resistance to Ignite® herbicide

Recipient organism - Maize (*Zea mays*)

Donor organisms - Cauliflower Mosaic Virus
- *Escherichia coli*
- *Streptomyces hygroscopicus*
- *Agrbacterium tumefaciens*

Vectors and DNA sequences used -

pDPG165: 35S 5' from CaMV
bar from *Streptomyces hygroscopicus*
Tr7 3' end from *Agrobacterium tumefaciens*

pDPG208: 35S 5' from CaMV
uidA (GUS) from *Escherichia coli*
NOS 3' from *A. tumefaciens*

Method of transformation - microprojectile bombbardment

Field location - Kihei, HI

Purpose of experiment - Observe gene espression and cross pollinate plants containing the *bar* gene to DeKalb proprietary inbred lines.

Summary of experimental results - Pollinations to maize inbreds were made.
- Plants expressed herbicide resistance.
- Normal Mendelian segregation observed.

Plant Characters Monitored for Changes:

Reproductive traits - no change
Disease status - no change
Morphological traits - no change
Herbicide resistance - now resistant to Ignite (aka Basta)
Weediness - no change
Ability to outcross - no change
Survival of progeny in the field - normal
Insect resistance - no change

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

Potential Field Containment Problems That were Monitored:

Wildlife or livestock invading field - none
Insect or weed control - normal
Adequate Isolation - was maintained
Water management - irrigation, normal rainfall

Disposition of volunteer plants after harvest: The fallowed fields were irrigated for three irrigation-tillage cycles to stimulate germination of corn seeds present in the soil. All volunteer plants were destroyed prior to anthesis.

Emergencies during field test? None

Changes in termination of the experiment from the method described in the application? None

Public reactions to the release: None

TERMINATION REPORT FOR: Field Release Permit #90-332-04

Period of release - May 24, 1991 - November 15, 1991

Trait - Resistance to Ignite® herbicide

Recipient organism - Maize (*Zea mays*)

Donor organisms - Cauliflower Mosaic Virus
- *Escherichia coli*
- *Streptomyces hygroscopicus*
- *Agrobacterium tumefaciens*

Vectors and DNA sequences used -

pDPG165: 35S 5′ from CaMV
bar from *Streptomyces hygroscopicus*
Tr7 3′ end from *Agrobacterium tumefaciens*

pDPG208: 35S 5′ from CaMV
uidA (GUS) from *Escherichia coli*
NOS 3′ from *A. tumefaciens*

Method of transformation - microprojectile bombbardment

Field location - DeKalb, IL

Purpose of experiment - Observe gene espression.

Summary of experimental results - Plants expressed herbicide resistance.
- Normal Mendelian segregation was observed.

Plant Characters Monitored for Changes:

Reproductive traits - no change
Disease status - no change
Morphological traits - no change
Herbicide resistance - now resistant to Ignite (aka Basta)
Weediness - no change
Ability to outcross - no change
Survival of progeny in the field - normal
Insect resistance - no change

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

Potential Field Containment Problems That were Monitored:

Wildlife or livestock invading field - none
Insect or weed control - normal
Adequate Isolation - was maintained
Water management - normal rainfall

Disposition of volunteer plants after harvest: The field was planted with soybeans in 1992 and monitored for volunteer plants. All volunteer plants were destroyed before they shed pollen.

Changes in termination of the experiment from the method described in the application? None

Public reactions to the release: None

TERMINATION REPORT FOR: Field Release Permit #91-317-01 (Renewal of Permit #90-332-02)

Periods of release - March 10, 1992 through June 4, 1992
- April 2, 1992 through July 1, 1992
- June 15, 1992 through August 28, 1992
- July 10, 1992 through October 14, 1992
- September 4, 1992 through November 19, 1992
- November 28, 1992 through March 15, 1993
- December 5, 1992 through March 24, 1993

Trait - Resistance to Ignite® herbicide

Recipient organism - Maize (*Zea mays*)

Donor organisms - *Escherichia coli*
- Cauliflower Mosaic Virus
- *Streptomyces hygroscopius*
- *Agrbacterium tumefaciens*

Vectors and DNA sequences used -

pDPG165: 35S 5′ from CaMV
bar from *Streptomyces hygroscopicus*
Tr7 3′ end from *Agrobacterium tumefaciens*

pDPG208: 35S 5′ from CaMV
uidA (GUS) from *Escherichia coli*
NOS 3′ from *A. tumefaciens*

Method of transformation - microprojectile bombbardment

Field location - Kihei, HI

Purpose of experiment - Observe gene espression and cross pollinate plants containing the *bar* gene to inbred lines.

Summary of experimental results - Pollinations to maize inbreds were made.
- Plants expressed herbicide resistance.
- Normal Mendelian segregation was observed.

Plant Characters Monitored for Changes:

Reproductive traits - no change
Disease status - no change
Morphological traits - no change
Herbicide resistance - now resistant to Ignite (aka Basta)
Weediness - no change
Ability to outcross - no change
Survival of progeny in the field - normal
Insect resistance - no change

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

Potential Field Containment Problems That were Monitored:

Wildlife or livestock invading field - none
Insect or weed control - normal
Adequate Isolation - was maintained
Water management - irrigation, normal rainfall

Disposition of volunteer plants after harvest: The fallowed fields were irrigated for three irrigation-tillage cycles to stimulate germination of corn seeds present in the soil. All volunteer plants were destroyed prior to anthesis.

Emergencies during field test? None

Changes in termination of the experiment from the method described in the application? None

Public reactions to the release: None

TERMINATION REPORT FOR: Field Release Permit #92-034-01 (Renewal of 90-332-04)

Period of release - May 29, 1992 - November 15, 1992

Recipient organism - Maize (*Zea mays*)

Donor organisms - Cauliflower Mosaic Virus
- *Escherichia coli*
- *Streptomyces hygroscopicus*
- *Agrobacterium tumefaciens*

Vectors and DNA sequences used -

pDPG165: 35S 5' from CaMV
bar from *Streptomyces hygroscopicus*
Tr7 3' end from *Agrobacterium tumefaciens*

pDPG208: 35S 5' from CaMV
uidA (GUS) from *Escherichia coli*
NOS 3' from *A. tumefaciens*

Method of transformation - microprojectile bombbardment

Field location - DeKalb, IL

Purpose of experiment - Observe gene espression and cross pollinate to maize lines.

Summary of experimental results - Plants expressed herbicide resistance.
- Normal Mendelian segregation observed.
- Cross pollinations were made.

Plant Characters Monitored for Changes:

Reproductive traits - no change
Disease status - no change
Morphological traits - no change
Herbicide resistance - now resistant to Ignite (aka Basta)
Weediness - no change
Ability to outcross - no change
Survival of progeny in the field - normal
Insect resistance - no change

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

Potential Field Containment Problems That were Monitored:

Wildlife or livestock invading field - none
Insect or weed control - normal
Adequate Isolation - was maintained
Water management - normal rainfall

Disposition of volunteer plants after harvest: The field was planted with soybeans in 1993 and monitored for volunteer plants. All volunteer plants were destroyed before they shed pollen.

Changes in termination of the experiment from the method described in the application? None

Public reactions to the release: None

Environmental Release Report

Permit Number: 93-014-02

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

Date of Release: April, 1993

Site of Release: DEKALB Plant Genetics Research Stations in Atlantic, IA (Cass County), Dayton, IA (Webster County), North Liberty, IA (Johnson County), Spencer , IA (Clay County), DeKalb, IL (DeKalb County), Mason City, IL (Mason County), Thomasboro, IL (Champaign County), Windfall, IN (Tipton County), Garden City, KS (Haskell County), Glenvil, NE (Adams County), Lima, OH (Allen County), Manheim, PA (Lancaster County), Dumas, TX (Moore County).

Purpose of Release: Mason County, IL and Champaign County, IL: evaluate agronomic performance of glufosinate resistant hybrids. Remaining sites: demonstration plots.

Results: Agronomic performance evaluated, glufosiante resistant corn demonstrated to farmers.

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:	no change

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

February, May, June, 1994
(Field tests were completed under APHIS notification 94-081-05N)

Means of Plant Disposition: Plants were repeatedly disked into the soil.

Monitoring for Volunteers: The fallowed fields were irrigated and tilled to stimulate germination of corn seeds present in the soil. All volunteer plants wree 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. Glufosinate resistant plants were tassel-bagged prior to anthesis and only controlled pollinations were made.

Ear shoots of all corn plants, transgenic and non-transgenic, within 660 feet of transgenic plants shedding pollen were ear bagged. No open pollinated corn was harvested and all harvested ears were used as part of a breeding program. Following harvest all fields are disked under and the field monitored for volunteers during a fallow period with irrigation.

In the April, 1993, nursery eleven transformants containing the *bar* gene were compared for level of resistance to glufosinate. Only two lines exhibited low level resistance to glufosinate. The remaining lines all exhibited resistance to up to 2.3 lbs active ingredient/acre (approximately seven times the recommended field application rate). The most common negative effects were slight stunting of plants and yellowing of the whorl. Two transformed lined were chosen for advance that demonstrated no adverse effects of herbicide application, even at the highest application rate. Eleven additional transformants were screened in the October, 1993, nursery for resistance to glufosinate and two lines were chosen for advancement.

In the nurseries planted in April, July, and October, 1993, and February and March, 1994, the selected transformants were backcrossed to 87 different elite DEKALB inbreds in order to introgress the *bar* gene into elite inbred lines.

Environmental Release Report

Permit Number: 92-365-04

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

Date of Release: Five releases: April, 1993; July, 1993; October, 1993; February, 1994; March, 1994

Site of Release: DEKALB Genetics research station located in Kihei, Maui County, Hawaii.

Purpose of Release: Crossing and self-pollinating of plants in order to introgress the *bar* gene into elite inbreds in all nurseries; evaluation of glufosinate resistance in eleven *bar* transformants in each of the April and October, 1993 nurseries.

Results: One transformant was selected in the April, 1993, nursery for advancement and two transformants were selected for advancement in the October, 1993, nursery. In all other nurseries, these lines were backcrossed to DEKALB elite inbreds.

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:	no change

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:	normal
Adequate Isolation:	was maintained
Water management:	irrigation

Date of Release Termination: July and October, 1993;

Monitoring of Field Containment

Wildlife or livestock invading field:	none
Insect or weed control:	normal
Adequate Isolation:	was maintained
Water management:	normal rainfall

Date of Release Termination: November, 1993

Means of Plant Disposition: Plants were repeatedly disked into the soil.

Monitoring for Volunteers: The fields were planted with a crop other than corn in 1994 and monitored for volunteer plants. All volunteer plants were destroyed before they shed pollen.

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. In agronomic performance tests transgenic corn plants were open pollinated and spatially isolated from other corn plants by at least 660 feet. Transgenic corn plants growing in demonstration plots were detasselled. There was minimal chance of outcrossing of transgenic corn to other corn.

Agronomic performance testing indicated that glufosinate resistant plants were equivalent in all traits examined to glufosinate sensitive plants, except for the additional trait of herbicide resistance.

The transgenic corn plants exhibited excellent resistance to glufosinate. Resistance segregated as a single Mendelian gene.s

95-145-01

Petition for Determination of Nonregulated Status:

Glufosinate Resistant Corn Line B16

DG# 95-128P

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

Submitted by:

Christopher E. Flick, Ph.D.
Program Manager, Discovery Research

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May 24, 1995

Contains No Confidential Business Information