AgrEvo

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97-265-01p

NO CBI

Mr. Michael A. Lidsky
Deputy Director, BBEP, APHIS, USDA
Att. BCPA
4700 River Road, Unit 146
Riverdale, MD 20737-1237

Re: Petition for Determination of Nonregulated Status for *Bt* CRY9C INSECT RESISTANT, GLUFOSINATE TOLERANT CORN TRANSFORMATION EVENT CBH-351

September 12, 1997

Dear Mr. Lidsky:

AgrEvo USA is submitting a Petition for Determination of Nonregulated Status for *Bt* CRY9C INSECT RESISTANT, GLUFOSINATE TOLERANT CORN TRANSFORMATION EVENT CBH-351. This petition requests a determination from APHIS that the corn transformation event CBH-351, and any progeny derived from crosses of event CBH-351 with traditional corn varieties, and any progeny derived from crosses of event CBH-351 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under CFR Part 340. Corn transformation event CBH-351 has been field tested widely across the corn belt in the United States and in Canada, Belgium, France, Chile and Argentina. The copies of the final termination reports for the trials conducted in the US are included in this petition.

Two copies of the petition are included which contain Confidential Business Information highlighted on pages 2 and 37. One copy of a Confidential Business Information deleted version is also included. Please find a Confidential Business Information Justification.

Should you have any questions, please feel free to contact me at (515) 276-6642.

Yours Sincerely,

Susan Mac Intosh

Susan C. MacIntosh

Product Safety Manager
AgrEvo USA

AgrEvo USA Company
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Received 9/22/97

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97-265-01p
NO CBI

Petition for Determination of Nonregulated Status

Bt Cry9C Insect Resistant, Glufosinate Tolerant Corn Transformation Event CBH-351

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

Submitted by

Susan C. MacIntosh
Regulatory Affairs Manager

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September 12, 1997

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Received
9/22/97

SUMMARY

AgrEvo USA Company is submitting a Petition for Determination of Nonregulated Status to the Animal and Plant Health Inspection Service (APHIS) for *Bt* Cry9C Insect Resistant, Glufosinate Tolerant Corn Transformation Event CBH-351. AgrEvo USA requests a determination from APHIS that corn transformation event CBH-351, and any progeny derived from crosses of event CBH-351 with traditional corn varieties, and any progeny derived from crosses of event CBH-351 with transgenic corn varieties that have also received a determination of nonregulated status, no longer be considered regulated articles under 7 CFR Part 340. Event CBH-351 is considered a regulated article because it contains sequences from the plant pests Cauliflower Mosaic Virus and *Agrobacterium tumefaciens.*

To provide a more reliable insect control method, Plant Genetic Systems N.V. (PGS), wholly owned by AgrEvo GmBh in Frankfurt Germany, has developed corn [*Zea* mays] plants that express an insecticidal protein, Cry9C from a common soil bacterium, *Bacillus thuringiensis* subsp. *tolworthi* (*Bt tolworthi*). The Cry9C protein is effective in controlling lepidopteran larvae such as European corn borer [*Ostrinia nubilalis* (Huber)] larvae, which are a common pest of corn.

In addition to the insecticidal gene, the chimeric *bar* gene which encodes the enzyme phosphinothricin acetyltransferase is also present in event CBH-351. The *bar* gene was isolated from *Streptomyces hygroscopicus*, a non-pathogenic bacterium. The integration of the *bar* gene enables the selection of the insecticidal line independent of the plant stage and provides tolerance to glufosinate-ammonium herbicides. The phosphinothricin acetyltransferase protein does not contain pesticidal activity and does not have any adverse environmental or toxicological effect.

Event CBH-351 has been field tested by several partners of AgrEvo since 1995 in the primary corn growing regions of the United States. These tests have occurred under field release authorizations granted by APHIS (USDA Notification Numbers 94-339-05n, 95-087-05n, 95-097-01n, 95-101-12n, 95-107-10n, 96-103-03n, 96-107-04n, 96-143-03n) CBI (*95-089-04n, 95-094-03n, 95-261-07n, 95-272-08n, 95-284-13n, 95-320-02n, 96-059-02n, 96-075-04n, 96-086-07n, 96-094-16n, 96-100-05n, 96-102-02n, 96-108-09n, 96-115-05n, 96-115-09n*). Corn transformation event CBH-351 has also been field tested in Canada, Belgium, France, Chile and Argentina. For the 1997 season, the CBH-351 event is being tested under an Experimental Use Permit, Number 70218-EUP-1. This EUP was approved by the Environmental Protection Agency on February 5, 1997 and covers expanded testing on up to 3,305 acres across 28 states/territories.

Data collected from these trials, laboratory analyses, reports, and literature references presented herein demonstrate that transformation event CBH-351: 1) exhibits no plant pathogenic properties; 2) is no more likely to become a weed than non-transgenic corn; 3)

likely to increase the weediness potential of any other cultivated plant or native wild es; and 4) is unlikely to harm other organisms that are beneficial to agriculture.

nsformation event CBH-351 was selected for commercial development. It has been ed with both public and proprietary inbred lines. A pesticide petition for an mption from the Requirement of a Tolerance and a Registration application for the t-pesticide: *Bacillus thuringiensis* subsp. *tolworthi* Cry9C and the genetic material ssary for the production of this protein in corn, CBH-351, was submitted to the EPA pril 4, 1997.

vo USA requests a determination from APHIS that the corn transformation event -351, and any progeny derived from crosses of event CBH-351 with traditional corn ties, and any progeny derived from crosses of event CBH-351 with transgenic corn ties that have also received a determination of nonregulated status, no longer be dered regulated articles under CFR Part 340.

CERTIFICATION

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

Susan C. MacIntosh
Regulatory Affairs Manager

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ABBREVIATIONS

Bt - *Bacillus thuringiensis*
CaMV - Cauliflower Mosaic Virus
CBH-351 - Transformation event that confers insecticidal and herbicidal tolerance
Cry9C - Insecticidal crystal protein from *Bacillus thuringiensis* subsp. *tolworthi*
cry9C - gene that encodes the Cry9C insecticidal protein
DBM - Diamondback Moth
ECB - European Corn Borer
ELISA - Enzyme Linked Immunosorbent Assay
HFCS - High Fructose Corn Syrup
IPM - Integrated Pest Management
IRM - Insect Resistance Management
NCR - Northern corn rootworm
PAT - Phosphinothricin acetyltransferase enzyme
bar - Phosphinothricin acetyltransferase gene
bla - β-lactamase gene
PPT - Phosphinothricin
PCR - Polymerase Chain Reaction
SWCB - Southwestern Corn Borer
WCR - Western Corn Rootworm

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Annex 4. Jansens, S., Van Vliet, A., Dickburt, C., Buysse, L., Piens, C., Saey, B., De Wulf, A., Paez, A., Gobel, E. And Peferoen, M. Field evaluation of transgenic corn expressing a Cry9C insecticidal protein from *Bacillus thuringiensis*, protected from European corn borer. In press *Crop Science*.

Annex 5. Composition of Grain from Cry9C Corn Derived from Transformation Event CBH-351

Annex 6. Molecular characterization of transformation event CBH-351

Annex 7. Letter of Dr. Marlin Rice (Iowa State University, Ames, IA)

Annex 8. USDA field trial termination reports

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1. RATIONALE

Plant Genetic Systems N.V., wholly owned by AgrEvo GmBh in Frankfurt Germany, has developed a line of corn [*Zea mays* L.] plants that express an insect control protein, Cry9C, from a common soil bacterium, *Bacillus thuringiensis* subsp. *tolworthi.* This transgenic corn event is known as event CBH-351. The Cry9C protein is very effective in controlling lepidoptera larvae, such as European corn borer [*Ostrinia nubilalis* (Huber)], which is a common pest of corn. European corn borer damage currently costs growers 5-20% yield loss. When chemical insecticide sprays are used, the efficacy is only marginal, due to the biology of the pest species. Transgenic corn plants expressing Cry9C protein represent an excellent addition to growers' options for insect control that reduces or eliminates the need for chemical inputs and fits well within an integrated pest management (IPM) program.

The transgenic corn event, known as *Bt* Cry9C Corn event CBH-351, also contains the *bar* gene, which encodes the phosphinothricin acetyltransferase (PAT) protein. The *bar* gene was isolated from the bacterium *Streptomyces hygroscopicus* (Thompson *et al.*, 1987) and gives a selective advantage to the transformed plants when they are sprayed with glufosinate-ammonium (De Block *et al.*, 1987). Use of glufosinate-ammonium herbicides in combination with *Bt* Cry9C Corn event CBH-351: 1) allows additional options for weed control, 2) can reduce the use of more toxic herbicidal compounds and substitute use of the more benign glufosinate-ammonium, 3) can reduce the overall amount of insecticides and herbicides a grower uses in corn production, 4) is compatible with IPM techniques, and 5) provides an additional tool for insect resistance management.

2. RECIPIENT ORGANISM : CORN (*ZEA MAYS* L.)

A. Production and Usage of *Zea mays*

Corn (*Zea mays* L.) is one of the major cereal grains grown in the world, being exceeded only by rice and wheat in terms of quantity produced. World corn production now normally exceeds 400 million tonnes, with US total production accounting for more than half of that for the entire world. Corn is the major crop on the cultivated land of the United States. It is produced on 75 to 85 million acres annually and plays an important role in the economy of the country. Although corn is produced throughout the United States, the bulk of US production occurs in the region known as the Corn Belt : two states, Iowa and Illinois, produce about 40 percent of the corn crop in the US (Jugenheimer, 1976; Hallauer *et al.*, 1988; Olson and Sander, 1988; Hallauer, 1994).

Relatively little corn is consumed directly as human food. When processed into meat, milk, eggs and other animal products, as three-fourths of it is, corn becomes the basic food plant of the modern American civilization. As indicated in Table 2.1., more than 75% of domestic corn is used for the feeding of livestock. Since corn generally has a low quantity and quality of protein for animal feed, it is usually complemented in feed rations with high quality-quantity protein sources such as soybean meal. Industrial utilization of corn accounts for about 20% of domestic maize consumption, either as starch *per se* or converted into products such as High Fructose Corn Syrup (HFCS), alcohol or glucose/dextrose. HFCS is currently used in most sweet drinks and snack foods. In the United States, direct human consumption of whole kernel or processed corn is limited (2-3%) and primarily derived from specialty corns such as white, pop and sweet corn (Mangelsdorf, 1974; Watson, 1988; Duvick, 1993; Rhoades, 1993; Hallauer, 1994).

Table 2.1. Corn usage in the United States (1991-1992) (Source : Duvick, 1993)

	Million tonnes	**(% domestic use)**	**(% industrial use)**
Feed	**124.39**	77	
Cattle and other	63.01	39	
Hogs	38.14	24	
Poultry	23.24	14	
Food, seed, industrial	**36.42**	**23**	
Corn syrup (HFCS)	9.96	6	30
Fuel alcohol	9.60	6	29
Starch	6.02	4	18
Glucose, dextrose	5.33	3	16
Beverage alcohol	2.08	1	
Cereal, other products	2.95	2	
Seed	0.51	0.3	
Exports	**40.23**		
Total use	**201.03**		

B. Biology of *Zea mays*

1. Taxonomy and genetics of *Zea mays* and its close relatives

Zea mays L. (2n=20) is a member of the family *Poaceae*, commonly known as the grass family, tribe Maydeae (Kiesselbach, 1980). Although there is not consensus on the origin and early evolution of corn, reasonably complete agreement exists among experts that corn was first domesticated in 8000 to 5000 B.C. in tropical south-central or south-western Mexico (Troyer, 1994). Corn is only known as a domesticated species. It can not reproduce itself successfully without the aid of man (Jugenheimer, 1976).

Teosinte is the closest relative of corn. There are three taxa of teosinte : *Zea mexicana* (Schrader) O. Knutze 2n=20, the annual diploid of wide distribution in Mexico and Guatemala; *Zea perennis* (Hitchcock) Reaves and Mangelsdorf 2n=40, the tetraploid perennial form now extinct in the wild; and *Zea diploperennis* Iltis, Doebley & Guzman 2n=20, the diploid perennial form found in a single locality, El Chante in Jalisco. Alternative taxonomy to that used here can be found in the literature (Wilkes, 1982). All three taxa can hybridize with corn; the F_1 hybrid from diploid parents is both robust and fertile. Teosinte is not native to the US. Though it is known to have survived as an escape from cultivation in Florida and Texas, teosinte is not considered a serious weed. It has been argued that the survival characteristics of teosinte as a wild plant are damaged by introgression from corn (Galinat, see letter in Annex 1).

The genus *Tripsacum* is the second closest relative of corn. Most species (13 to 16 different species are recognized) are native to Mexico, Central and South America. *T. floridanum* Porter ex Vassy 2n=36, however, is native to Southern Florida. *Tripsacum. dactyloides* (Eastern gamagrass) 2n=36 is native to the central and western US, and the tetraploid form of *T. dactyloides* 2n=72 extends along the Eastern seaboard from Massachusetts to Florida and along the Gulf Coast from Florida to Texas. *T. lanceolatum* 2n=72 is a tetraploid that occurs in the Southwestern US. There is no evidence for natural hybridization between corn and *Tripsacum* in North America. F_1 hybrids (male sterile) have been obtained with varying degrees of difficulty under experimental conditions only. *Tripsacum* is not considered an aggressive weed. Recently, there has been a growing demand for *T. dactyloides* seed for planting as a new forage or haylage crop in the Great Plains area of the US (Galinat, see letter in Annex 1.; Kindiger, see letter in Annex 2.).

2. Cultivation of *Zea mays*

As with all commercially grown crops, optimal growth conditions are essential to assure a high yielding corn crop. Although corn can be grown under a wide range of conditions, particular attention is required to optimum planting procedures, a balanced fertilization of the plants, seed selection, weed control and pest and disease control.

2.a. General methods

Corn is a summer row crop, appreciating a warm but not excessively hot environment for growth. Most agronomists agree that the optimum time for planting corn is as soon as the soil temperature (at 5 cm depth) reaches a minimum of 10°C (50°F) for a relatively sustained period of time. Since the mid-1970s, there has been a major trend away from extensive conventional primary and secondary tillage towards reduced or no tillage. A combination of several factors has been responsible for this evolution, most importantly the advent of effective herbicides for weed control, the benefits from a residue cover in erosion control and the obvious economic advantages of reduced implement usage. Other trends include increasing plant densities and narrower row spacing. Corn has a high nitrogen demand and substantially more nitrogen is used for its production than any other primary fertilizer nutrient such as phosphorous, potassium and sulfur. With the exception of a few unique conditions and/or localized areas, addition of other elements are usually considered unnecessary (Hallauer *et al.*, 1988; Olson and Sander, 1988; Wych, 1988).

2.b. Weed control practices

Corn is not considered to be a strong competitor (Keeler, 1989). Uncontrolled weeds can easily cause a complete loss of corn yield and even small numbers of weeds can substantially reduce the yield of the crop (Olson and Sander, 1988). Some of the major weeds of corn are listed in Table 2.2..

Table 2.2. Major corn weeds (FAO, 1982)

Grasses	Broadleafs
Giant foxtail	Cocklebur
Green foxtail	Lambsquarter
Yellow foxtail	Mustard
Barnyard-grass	Pigweed
Crabgrass	Ragweed
Panicum	Smartweed
Wooly cupgrass	Velvetleaf
Wild proso millet	Wild sunflower
Nutsedge	Canada thistle
Quackgrass	

It is essential that early weed control is achieved after emergence either through tillage (rotary hoe or harrow) or via chemical treatment. Later weeds are often controlled by cultivation, most commonly with shovel cultivators.

Though a combination of physical, mechanical and chemical methods is available to control weeds in corn, herbicides remain an important means of weed control. Without herbicides, some reduced and no-till systems of corn production would be doomed to failure. In recent years, more environmental friendly herbicides have been developed for specific weed problems and specific cropping and tillage systems. Since new herbicides

and new combinations of herbicides are constantly being developed, please reference the most recent Agricultural Extension publications for a list of currently used herbicides in *Zea mays* (Olson and Sander, 1988).

2.c. Insect control practices

The corn crop is subject to attack by a complex of insects from the time it is planted until it is utilized as a food or feed (see Dicke and Guthrie, 1988 for an extensive review). Two of the most important insect pests in the U.S. are the European corn borer (*Ostrinia nubilalis* (Hübner)) and the corn rootworms (*Diabrotica* spp.), both of which can especially be a problem in corn-after-corn crop rotations. Approximately 25-35% of all corn acres have traditionally been treated with some type of insecticide.

European corn borer (ECB) is a major pest of maize in North America. Yield losses of 3-7% per borer per plant can result from ECB feeding at various stages of plant growth. Evaluating germplasm for corn borer resistance by manual infestation of artificially-reared ECB eggs is a typical part of a commercial corn breeding program. Increased levels of ECB resistance contribute to improved plant health and has been a factor contributing to the genetic gains experienced in newer hybrids. Resistance is not absolute, however, and hybrids vary widely in their degree of resistance. Thus, insecticides are still a common control measure for ECB. Annual treatment costs and annual crop losses for ECB are estimated at $50 million and $1 billion respectively (Lynch, 1980; Lewis, 1991).

The two most important *Diabrotica* pests are *D. virgifera virgifera*, the western corn rootworm (WCR) and *D. barberi*, the northern corn rootworm (NCR). These pests have caused an estimated 10-13% yield loss per year. WCR and NCR are mainly found in the Northern U.S., east of the Rocky Mountains. The WCR has become the dominant rootworm pest in a large area of the Corn Belt. WCR and NCR are particularly a serious problem where continuous corn is grown. Consequently, the practice of short rotation, particularly with an intervening crop of soybean, has become common in the Corn Belt. Where continuous corn is practiced, several effective insecticides are available to ensure against appreciable yield losses; approximately 50-60% of the corn acreage is treated with soil insecticides. Treatment costs and crop losses are in the range of $1 billion per year (Metcalf, 1986).

Though the economic losses from ECB are large, treatment for ECB has been limited because the pest has been difficult to control in an effective manner. The life cycle of the insect requires that timing and the location of treatment applications be precise. The treatment must be applied after the eggs have hatched and while active feeding is occurring, and before the ECB larvae get too large and burrow into the stalk. Otherwise, treatments may be completely ineffective. For this reason, chemical control for ECB has been used most extensively in geographic areas that routinely experience high levels of ECB infestation, such as eastern Nebraska. Corn seed production fields are also commonly treated for ECB control, due to the relatively high value of the seed crop being grown. Though losses from ECB extend far beyond these acres currently treated, the

unpredictability of pest damage and the difficulty in obtaining effective control have hampered more extensive efforts to control the pest.

Yield losses caused by ECB in corn can be the result of two different mechanisms. The first is from physiological damage, caused by the ECB tunnels disrupting the flow of water, nutrients, and photosynthate through the plant. The second mechanism is from harvest losses, due to stalk breakage and dropped ears as a result of tunneling. Of the two, the physiological damage is much greater in reducing yields, but more difficult to measure and document. The only method currently available to control the physiological damage is the application of pesticides. To reduce harvest losses, corn growers may choose to harvest crops with ECB damage earlier in the season. However, this does nothing to prevent the physiological damage. There is also an additional expense associated with early harvest, because grain harvested earlier will be higher in moisture and requires addition expenditures for artificial drying of the grain.

Certain cultural practices and a number of insecticides can be used to minimize or control insect damage. As the list of registered insecticides may change every year, publications can be checked annually for changes in recommendations (Dicke and Guthrie, 1988).

2.d. Disease control practices

Corn is rarely grown in the absence of diseases. Estimates made of disease losses for corn in the U.S. caused by all pathogens have ranged from as little as 2-7% to as much as 7-17% yearly. A wide range of organisms, including certain fungi, bacteria, viruses, nematodes, at least one mycoplasma, one spiroplasma and one parasitic seed plant are known pathogens of corn in the U.S. (see Smith and White, 1988 for an extensive review). These pathogens vary in their adaptability to specific environments and their geographic area of recognized occurrence, which also may shift over time (Smith and White, 1988).

Attempts to protect the corn crop from diseases include the use of chemical control measures, resistant cultivars and preventative cultural practices. The seed of commercial hybrid corn is treated with fungicides to help prevent losses from seed rots and seedling blights (e.g. *Pythium* spp.) that can occur if adverse environmental conditions occur shortly after planting. Although foliar fungicides are used on high-value corn crops such as seed production fields, commercial popcorn and sweet corn fields, currently no routine chemical control of diseases on grain or silage crops is commonly used. Most corn germplasm used in hybrids in the U.S. is being selected in regular corn breeding nurseries for acceptable levels of resistance to common diseases to which the resulting hybrids would be most likely exposed. A common cultural practice to control diseases in corn is crop rotation to reduce the perpetuation and intensification of corn pathogens which can occur with continuous cropping of corn (Smith and White, 1988).

3. TRANSFORMATION METHODOLOGY

The *Bt* Cry9C Corn transformation event CBH-351 contains a modified *cry9C* gene derived from an insecticidal crystal gene from *Bacillus thuringiensis* subsp. *tolworthi*, and the *bar* gene, derived from *Streptomyces hygroscopicus*. The *bar* gene encodes the PAT enzyme, which confers resistance to glufosinate ammonium herbicides. The *cry9C* gene is fused to a *35*S promoter and 3' terminator from CaMV, while the *bar* gene is linked to a *35*S promoter from CaMV and nos 3' terminator from *Agrobacterium tumefaciens*. The plasmids pRVA9909 and pDE110, for *cry9C* and *bar*, respectively, were used to transform the backrossed hybrid corn line (PA91 x H99) x H99 produced at Plant Genetic Systems. The plasmids contain no other plant expressible genes. The plasmids were transferred to the corn plant genome through direct plasmid DNA introduction using microprojectile bombardment. Stable integration of the *cry9C* and *bar* genes into the corn genome results in the production of the Cry9C insecticidal protein and the PAT enzyme in the transformed corn plants.

A. The Transformation System and Plasmids

1. Transformation method

As mentioned above, plant transformation was performed using microprojectile bombardment of corn tissue. Introduction of DNA into plant tissue via microprojectile bombardment is a well-known procedure. By shooting DNA coated gold particles into the cell, the membrane is permeated so that DNA molecules can be introduced into the cell (Vasil, 1994). By this method, the plasmids containing the transgenes were introduced into recipient tissues derived from public inbred lines of corn and from crosses between public inbred lines of corn (*Zea mays* L.).

Tissue microprojectile bombardment has been used for the co-delivery of two different plasmid DNAs, pRVA9909 and pDE110 (Figures 3.1. and 3.2., respectively). Transgenes on both plasmids were expressed, and co-segregated as a single dominant unit, indicating that the two DNAs were integrated at the same locus (Section 4.B., Genetic Stability).

2. Recipient Corn Line

H99 is an inbred line developed at Purdue University (West Lafayette, Indiana), from which it was released in 1974. It was produced by self-pollination in the population called Illinois Synthetic 60C, which in turn was developed by crossing the USDA Blight Resistant Double population with the inbred lines B8, Ia55:473, M14, Oh43, Oh45, Oh51A, R160 and R168 (personal communication with Dr. B. Zehr, Purdue University). H99 was chosen as the recipient line for the transformation procedure because of its superior qualities in tissue culture, particularly its high frequency of type I callus formation (Duncan et al., 1985; Hodges et al., 1986).

PA91 is an inbred line developed at Penn State University (State College, PA), from which it was released in the mid-70's. The pedigree of the line is ((WF9 x Oh40B[4]) x (L317 x Ind38-11[4])) (MBS, Inc., 1989) with a maturation period of 128 days.

3. The DNA elements of the plasmids

Transformation vectors suitable for introduction of the *cry9C* and the *bar* transgenes into plants have been developed by inserting each gene separately in the pUC19 cloning vector, using *E. coli* as a host (Yanisch-Perron *et al.*, 1985). pUC19 is a commonly used vector in *E.coli.* Plasmid pRVA9909 (Figure 3.1., pg. 21) contains the chimeric modified insecticidal gene (*cry9C.PGS2a*). The chimeric *cry9C.PGS2a* gene encodes a protein which corresponds to the insecticidal portion of the Cry9C protein produced by *Bt tolworthi*, BTS02618A (Annex 3., Lambert *et al.*, 1996). A summary of the DNA components are presented in Table 3.1.

Plasmid pDE110 (Figure 3.2., pg. 22) contains the chimeric *bar* gene. The chimeric *bar* gene encodes a phosphinothricin acetyltransferase enzyme which acetylates glufosinate and thereby detoxifies glufosinate. A summary of the DNA components are presented in Table 3.2.

Both plasmids also contain an origin of replication (*ori*) required for replication of the plasmids in *Escherichia coli*, and the ß-lactamase gene (*bla*) which confers resistance to antibiotics, such as ampicillin, in bacteria to insure retention of the plasmids. Both of these sequences are functional only in the *E. coli* bacterial host.

Table 3.1. Summary of DNA components in pRVA9909

GENETIC ELEMENT	SOURCE	POSITION ON VECTOR	FUNCTION
CaMV *35S*	Cauliflower mosaic virus	1-2	Promoter of the *35S* promoter of CaMV
polylinker derived sequences	synthetic	3-6, 67-72, 1951-1963, 2179-2182	cloning facilitation
cab22L	Petunia	7-66	Leader sequence of *cab22L* gene of petunia to enhance gene expression
cry9C	*Bacillus thuringiensis* subsp. *tolworthi*	73-1950	DNA fragment containing the coding sequence of the truncated insecticidal gene, *cry9C.PGS2a*
CaMV *35S*	Cauliflower mosaic virus	1964-2178	polyadenylation signal of the *35S* terminator of CaMV

pUC19	*Escherichia coli*	2183-3373, 4235-4620	DNA sequences from the high copy plasmid used for cloning of DNA sequences
bla	*Escherichia coli*	3374-4234	DNA fragment containing the ß-lactamase gene, which confers resistance to ampicillin in bacteria
3'*35S*	Cauliflower mosaic virus	4621-5145	Promoter of the *35S* promoter of CaMV

Table 3.2. Summary of DNA components in pDE110

GENETIC ELEMENT	SOURCE	POSITION ON VECTOR	FUNCTION
pUC19	*Escherichia coli*	1-396, 2615-3822, 4684-4883	DNA sequences from the high copy plasmid of pUC19, used for cloning of DNA sequences
CaMV *35S*	Cauliflower mosaic virus	397-1779	Promoter of the *35S* promoter of CaMV
bar	*Streptomyces hygroscopicus*	1780-2331	Phosphinothricin acetyltransferase gene for herbicidal tolerance
nos	*Agrobacterium tumefaciens*	2350-2610	A 3' nontranslated region of the nopaline synthase gene involved in transcription termination and polyadenylation
polylinker derived sequences	synthetic	2332-2349, 2611-2614	cloning facilitation
bla	*Escherichia coli*	3823-4683	DNA fragment containing the ß-lactamase gene, which confers resistance to ampicillin in bacteria

3.a. *CaMV 35S promoter and cab22L leader sequence*

The *35S* promoter sequence are derived from the Cauliflower Mosaic Virus and are used to control expression of both the *cry9C* and the *bar* genes (Odell *et al.* 1985). The *cab22L* leader sequence is derived from petunia. Both the *35S* promoter and *cab22L* leader (Harpster *et al.* 1988) is widely used in plant transformation to direct high level constitutive expression of genes. The CaMV and *cab22L* sequences, as used in *Bt* Cry9C corn event CBH-351, do not cause the corn to become a plant pest.

3.b. cry9C gene

The *cry9C* gene that encodes the insecticidal protein Cry9C was isolated from *Bt tolworthi*. A full length clone of the *cry9C* gene, the nucleotide sequence, the deduced

amino acid sequence, the sequence homology to other Cry toxins and the insecticidal spectrum for the Cry9C protein have been described in detail by Lambert *et al.* (1996). The coding sequence of the *cry9C* gene was modified to produce the *cry9C.PGS2a* coding sequence for use in plant transformation. The following changes have been introduced in the plant produced protein, as compared to the wild type Cry9C protoxin: 1) a C-terminal truncation that removes all the amino acids following position 666 of the wild type protoxin, 2) a N-terminal truncation that removes the first 43 amino acids and that adds of methionine - alanine, 3) a replacement of arginine by lysine at amino acid position 123 of the plant encoded protein. In pRVA9909 this mutation is present at position 439-441. The replacement of arginine by lysine reduces the susceptibility of the protein to trypsin cleavage, which may be responsible for the degradation of the protein to a nontoxic fragment. These changes do not appear to affect the insecticidal activity of the Cry9C protein (Lambert *et. al.*). For more detail about the Cry9C protein see section 6.A.

3. c. bar gene

In addition to the insecticidal *cry9C* gene, these corn plants also express the *bar* gene which encodes a PAT protein conferring tolerance to glufosinate-ammonium herbicides. The PAT protein acetylates glufosinate-ammonium and thereby detoxifies the herbicide. This gene product also provides a selectable marker trait in a breeding program with the insecticidal gene to identify which plants carry the insecticidal protein.

The *bar* gene encodes tolerance to herbicides with glufosinate as the active ingredient (e.g. Basta®, Buster®, Finale®, Ignite®, Challenge®, Harvest®, Liberty®; tradenames of AgrEvo). As an analogue of glutamate, glufosinate inhibits glutamine synthetase in plants. The inhibition of glutamine synthetase by glufosinate results in an accumulation of ammonium. In addition, a process connected with photorespiration plays a central role on photosynthetic inhibition by glufosinate (Wild *et al.*, 1984; Manderscheid *et al.*, 1985; Wild *et al.*, 1987; Sauer *et al.*, 1987; Wendler *et al.*, 1990). To protect the *Zea mays* plant against the toxic effects of the glufosinate compound, the *bar* gene can be incorporated into the plant genome, and expressed leading to the production of the enzyme phosphinothricin acetyltransferase. This enzyme acetylates glufosinate and inactivates the molecule, thereby preventing death of the plant cell (De Block *et al.*, 1987). For more detail about the PAT protein see section 6.B.

3.d. bla gene

The coding sequence for ß-lactamase, the *bla* gene, is present and confers resistance to ampicillin when in *E. coli*. It is used to select those bacterial cells that contain the transforming plasmids (Yanisch-Perron *et al.*, 1985). The expression of *bla* is controlled by a procaryotic promoter which is that functions only in procaryotes and not in eucaryotic plants.

Figure 3.1. Plasmid map of pRVA9909

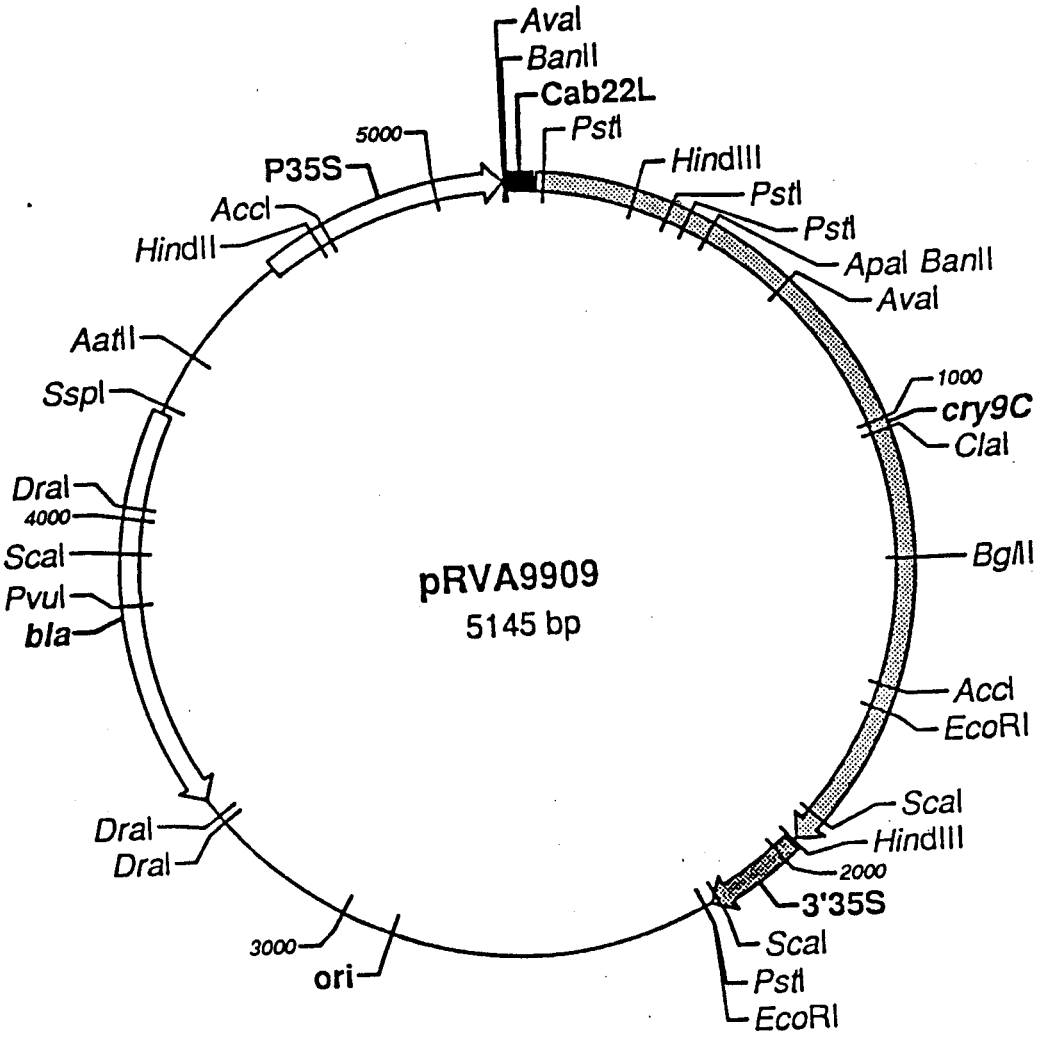
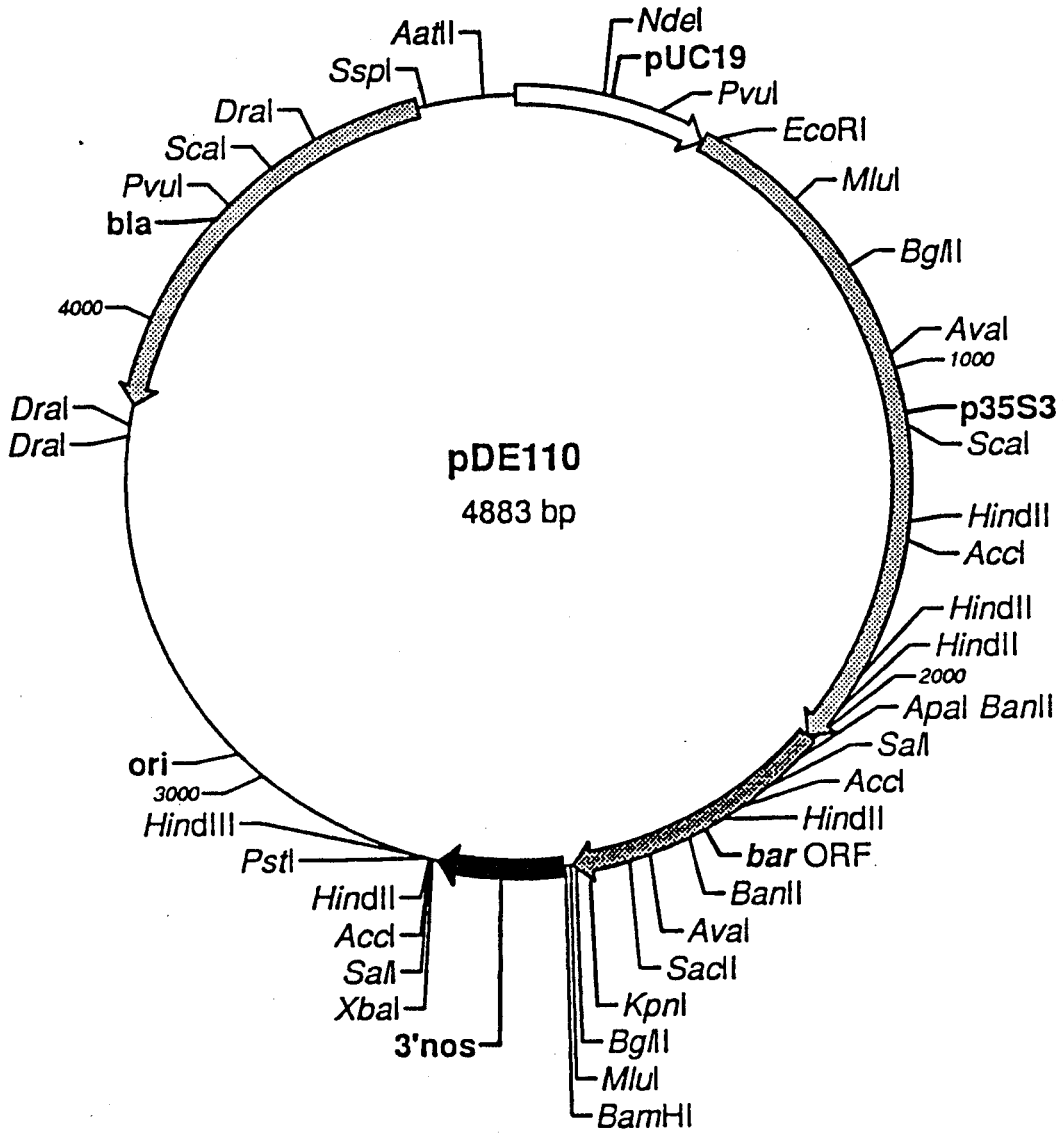


Figure 3.2. Plasmid map of pDE110



4. MOLECULAR AND BIOCHEMICAL CHARACTERIZATION OF THE *BT* CRY9C CORN EVENT CBH-351

The transformation event to be considered for non-regulated status has been designated CBH-351. The commercialization strategy in corn will be to use traditional backcrossing of elite inbred recipient plants with donor plants containing the CBH-351 event, in order to develop elite commercial inbreds, containing the CBH-351 event. These elite inbred parental lines will be used to produce commercial hybrid seed. Consequently, this petition requests non-regulated status for the CBH-351 event; not only in the donor or recipient inbred lines, but also in any corn genotype, having received non-regulated status.

Molecular analyses of the transgenic plants were carried out to determine the number of insertion sites, the number of copies of the transgenes, and the expression and genetic stability for the transgenes in the CBH-351 event. Plant material was sampled from field trials (see Section 5) for biochemical and molecular analyses. A summary is presented below and a detailed description of the analyses is provided in Annex 6. The short table below guides the reader to the location of the detailed data:

Gene	Protein	Is a complete copy present?	Is protein expressed in the plant?	Location of detailed data.
cry9C	Cry9C	yes	yes	Fig. 4.1, pg 24 Table 4.2-4.5, pg 32-35 Annex 6, pg 33
bar	PAT	yes	yes	Fig. 4.1, pg 24 Table 4.2-4.5, pg 32-35 Annex 6, pg 34
bla	ß-lactamase	yes	no	Annex 6, pg 32, 35

A. Gene Copy Number

During a series of experiments using Southern analysis, genomic DNA from transgenic plants of transformation event CBH-351 were digested by restriction enzymes. The DNA fragments were separated on a gel, transferred to a solid matrix and hybridized by specific probes. These molecular analyses demonstrated that at least one copy of the *cry9C* gene and four copies of the *bar* gene have been inserted at a single site in the corn genome for event CBH-351 (Figures 4.1. and 4.2.) The characterization of the insert is fully described in Annex 6. [refer to Annex 6, Material and Methods [pgs. 4-8], Results [pgs. 14-21], Figures 1-4).

Figure 4.1. Southern hybridization of DNA from the corn transformation event CBH-351. The gel was probed with radioactive labelled DNA fragments as indicated in A-D below. Plant DNA was digested with the restriction enzyme EcoRI (E), SspI (S), and both EcoRI and SspI (ES). Molecular weight markers are indicated at the right.

A. Probe: *cry9C* gene
B. Probe: *bar* gene.
C. Probe: 5' promoter region of the *35S* gene
D. Probe: plasmid pRVA9906 DNA, linearized with the restriction enzyme BamHI

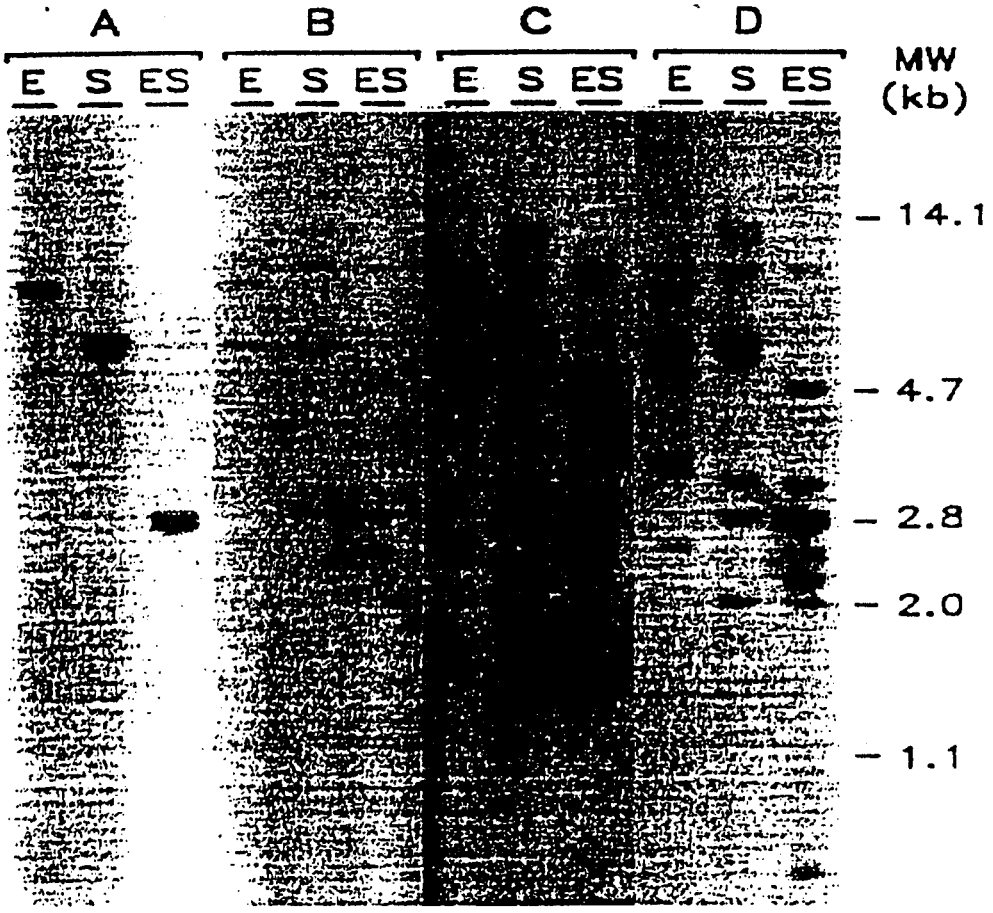
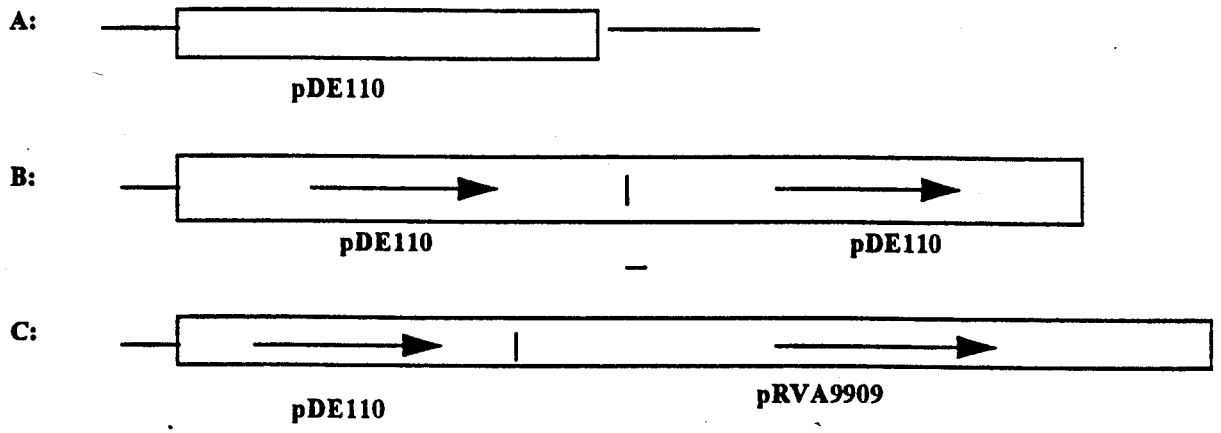


Figure 4.2. Schematic representation of the insertion of the *cry9C* and *bar* genes in event CBH-351



B. The Genetic Stability

The stability of the transferred DNA in the *Bt* Cry9C corn event CBH-351 was determined at the genetic (Southern analysis) and the phenotypic level (segregation and linkage analysis).

1. Evaluation of the genetic stability by Southern analysis

Southern analysis represents the most straightforward method to characterize the inserted DNA present in the plant genome. Plants from the various generations descended from the initial transformation event CBH-351 and offspring from CBH-351-derived hybrids from different genetic backgrounds were subjected to Southern analyses. The analysis demonstrated the stability of the inserted DNA over generations and in different genetic backgrounds.

Genomic DNA from different transformed plants was digested by the HindIII restriction enzyme. After separation of the DNA restriction fragments by electrophoresis, the gel-separated bands were blotted onto a solid matrix. The inserted DNA was characterized by hybridization with a specific radioactive labeled DNA probe complementary to the elements present in the inserted plasmid DNA. The banding pattern, or 'fingerprint', should be identical across generations and genotypes if the introduced inserted DNA is inherited as a stable genetic element and has not been rearranged in the plant genome during the plant breeding process.

A similar analysis has been performed to characterize stability of the inserted DNA for CBH-351-derived hybrids from different genetic backgrounds. The hybridization pattern of all analyzed offspring of the CBH-351-derived hybrids from different genetic backgrounds that contained the CBH-351 event and the pattern of the primary transformation event were identical, thus implying that the inserted DNA in corn transformation event CBH-351 is also stably inherited across different genetic backgrounds (Figure 4.3., also refer to Annex 6, Materials and Methods [pgs. 4-8], Results [pg. 22], Figure 10).

The plasmid pRVA9906 contains all functional elements present in the transformation vectors pDE110 and pRVA9909, within a single vector. This plasmid was used as the radiolabelled probe. The initial transformant (designated "To" in the figure) was crossed sequentially to H99 in a series of backcrosses to produce 5 different generations. The hybridization pattern for all of the analyzed offspring for the different generations and from the primary transformation event were identical, thus implying that the inserted DNA in corn transformation event CBH-351 is stably inherited across generations (Figure 4.4., also refer to Annex 6, Materials and Methods [pgs. 4-8], Results [pg. 23], Figure 11).

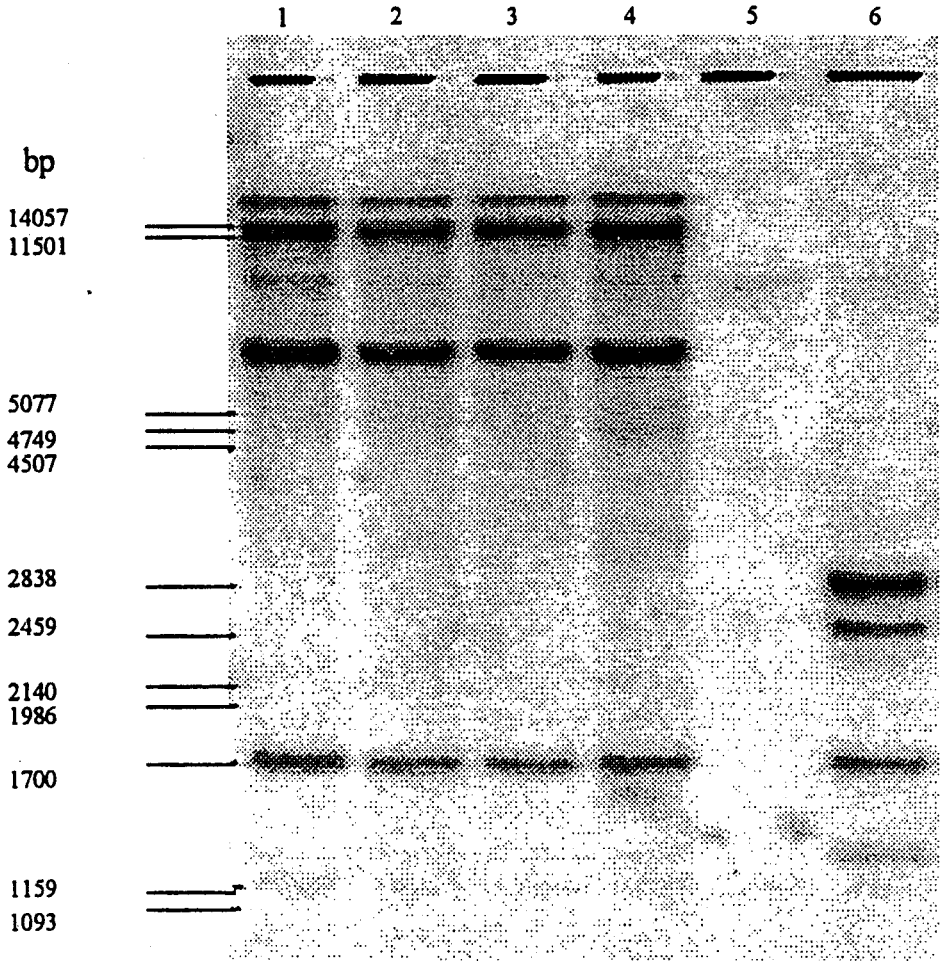


Figure 4.3: Southern Blot Analysis - Stability of the insert of CBH351 in different backgrounds

Genomic DNA digested with HindIII and probed with pRVA9905

1. (((B73 x (To x B73)) x Mo17);
2. (Mo17 x ((B73 x (To x B73)) x B73));
3. (A619 x ((B73 x (To x B73)) x B73));
4. (B73 x ((B73 x (To x B73)) x B73));
5. B73 wild type;
6. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

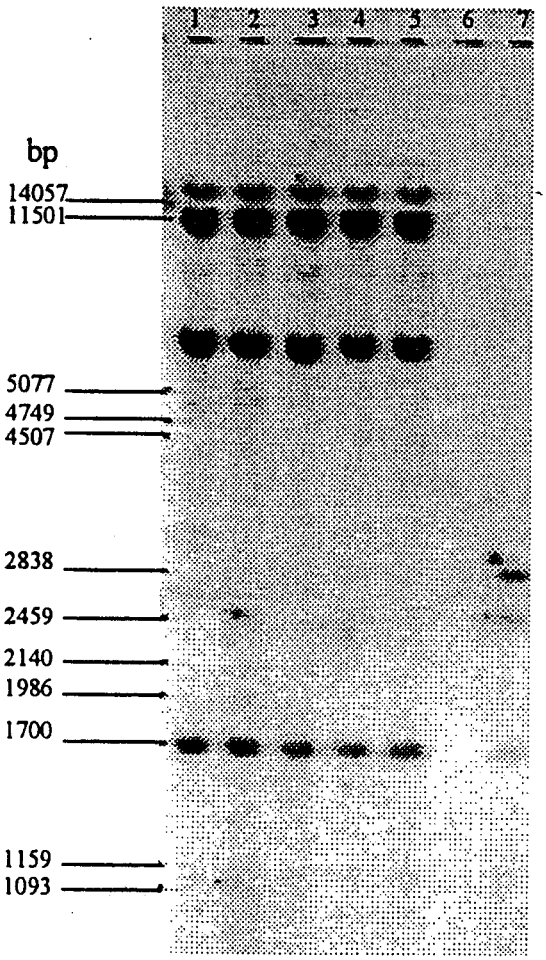


Figure 4.4: Southern Blot Analysis - Stability of the insert of CBH351 in different generations

Genomic DNA digested with HindIII and probed with pRVA9906
1. T1 (H99 x To). 2. T2 (H99^2 x To). 3. T3 (H99^3 x To). 4. T4 (H99^4 x To). 5. T5 (H99^5 x To). 5. B73 wild type. 6. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

2. Segregation and linkage analysis

The analysis for segregation of one or more traits in offspring from a cross with a particular parental line can evaluate the Mendelian inheritance of each trait and the linkage of the respective traits. In the case of transformation event CBH-351, a parental line hemizygous with respect to the inserted DNA fragment contains the glufosinate herbicide tolerance and the insect resistance traits. If the inserted DNA is present at a single locus in transformation event CBH-351, the offspring obtained from a cross between a non-transgenic line and the hemizygous corn CBH-351 line should segregate in a 50:50 ratio, resulting in 50% herbicide-tolerant / insect-resistant corn plants and 50% herbicide-sensitive / non-insect-resistant corn.

Such segregation analysis has been performed and a detailed description of the trial is presented in Annex 7. More than 2000 plants derived from a cross between the non-transgenic line A136 and a hemizygous CBH-351 line were analyzed for segregation of the herbicide tolerance trait (Table 4.1. below). Glufosinate tolerance analysis (Liberty® dot analysis) indicated that in each of the nine plots about 50% of the plants were herbicide resistant. If considered as a single large plot, with all plants combined, the 50:50 segregation ratio is also confirmed. In plots 1, 5, and 9, twenty-five herbicide tolerant plants were infested with ECB and scored for insect resistance. No damage on any of the infested herbicide-tolerant plants was observed, indicating that both herbicide tolerance and insect resistance are closely linked.

The segregation analysis of offspring from the cross between the hemizygous corn transformation event CBH-351 and a non-transgenic corn line for glufosinate tolerance and insect resistance illustrates that the herbicide tolerance and the insect resistance traits are present at a single locus in the transformation event CBH-351, and that the traits are transmitted to progeny in a Mendelian fashion as dominant traits.

Table 4.1. Segregation analysis for glufosinate-ammonium tolerance of CBH-351xA619 progeny

Plot #	# of Plants	Liberty Sensitive	Liberty Resistant	Sen./Tol. Expected	X^2 value
1	239	135	104	119.5	3.766
2	269	123	146	134.5	1.799
3	247	139	108	123.5	3.644
4	261	124	137	130.5	0.552
5	256	140	116	128	2.066
6	261	143	118	130.5	2.207
7	224	117	107	112	0.361
8	220	115	105	110	0.368
9	211	108	103	105.5	0.076
Total	2188	1144	1044	1094	4.479

C. The Pattern of Expression of the Transgenes

The efficiency of insect protection of transformation event CBH-351 will depend on the location of the insert and the level of the transcription and translation of the insecticidal *cry9C* gene. The transcriptional level of expression in a certain tissue is measured by Northern analysis, while a direct measure of the translational level in each tissue can be determined by protein-specific immunological ELISA.

It was the objective to obtain high Cry9C protein expression in all major tissues susceptible to ECB. For this, the expression of the synthetic *cry9C* gene has been linked to the constitutive and highly expression *35S* promoter of CaMV. Therefore, a high level expression of the Cry9C protein in all tissues is expected, except for pollen, where the transcription of the *35S* promoter has been reported to be quite low (Koziel, *et. al.*, 1993).

Herbicide tolerance to the herbicidal compound glufosinate will depend on an effective expression of a herbicide tolerance gene in the target tissues, including all upper tissues expressing glutamine synthase. For this, the expression of the synthetic *bar* gene was also linked to the constitutive and highly expression *35S* promoter of CaMV, and a high level expression of the PAT protein in all tissues is also expected, except for pollen (see above).

The bacterial *bla* gene, encoding a β-lactamase enzyme, was used as a selectable marker during cloning of the plasmids in *E.coli*. The expression of this gene is linked to bacterial transcription regulatory sequences, which are not recognized in plants. The *bla* gene is not expected to be expressed in the corn transformation event CBH-351.

1. Expression of the insecticidal *cry9C* gene

As expected from the above outlined expression strategy, the transferred *cry9C* gene is expressed in leaves, stem, and root tissues as determined by northern analysis (Annex 6, Results [pgs. 21], Figure 7). Evidently the *cry9C* mRNA level in seeds is below the detection limits of the analysis. The presence of a 3' polyadenylation tail on the isolated plant RNA causes a small increase in the size as compared to the *in vitro* synthesized RNA.

In order to determine the accumulation of Cry9C protein during a complete field season, a detailed ELISA analysis of the Cry9C protein has been performed over a complete growing season (Tables 4.2., 4.3.). The study was submitted to the EPA as part of the Registration Application for *Bt* Cry9C Corn. The Cry9C protein level was quite stable, with at most a 2-fold difference between the first two stages tested, vegetative growth and pollen shed. At later sampling stages in the season, silage and harvest, the levels of all proteins tended to decline (Tables 4.2.). When Cry9C protein level is expressed as a % of the total protein (Tables 4.3.), less than a 2-fold drop occurs between the first and fourth stages of sampling. The level of Cry9C protein in seeds is substantially less than that found in all other tissues. As such, the Cry9C protein still remains at a sufficiently high dose to be toxic to ECB.

To examine expression of the *cry9C* gene within different genetic backgrounds, a detailed ELISA analysis of the Cry9C protein levels in whole plants derived from hybrid CBH-351 plants in 4 different genetic backgrounds has been performed (Tables 4.4., 4.5.). The observed levels of the Cry9C protein were consistent for the respective whole plants, indicating that the *cry9C* gene is also stably expressed in different genetic backgrounds.

It is concluded that the *cry9C* gene present in transformation event CBH-351 will be expressed in all major tissues infested by ECB in hybrid corn plants, at a sufficiently high level to provoke toxicity towards the insect during the complete growing season. Moreover, the *cry9C* gene is stably expressed within different genetic hybrid corn backgrounds.

2. Expression of the herbicide tolerance *bar* gene

As expected from the above outlined expression strategy, the transferred *bar* gene is expressed in leaves, stem, and root tissues as determined by northern analysis (Annex 6, Results [pgs. 21], Figure 8). Evidently the *bar* mRNA level in seeds is below the detection limits of the analysis. The presence of a 3' polyadenylation tail on the isolated plant RNA causes a small increase in the size as compared to the *in vitro* synthesized RNA.

In order to determine the expression and accumulation of PAT protein, a detailed ELISA analysis of the PAT protein has been performed over a complete growing season (Tables 4.2., 4.3.). The PAT protein level was quite stable, with at most a 2-fold difference between the first two stages tested, vegetative growth through pollen shed (Table 4.2.). At later sampling stages in the season, silage and harvest, the levels of all proteins tended to decline. When PAT protein level is measured as a % of the total protein (Table 4.3.), approximately a 6-fold drop occurs between the first and fourth stages of sampling. The level of PAT protein in seeds is substantially less than that found in all other tissues.

In order to measure the expression of the *bar* gene within different genetic backgrounds, a detailed ELISA analysis of the PAT protein levels in whole plants derived from hybrid CBH-351 plants in 4 different genetic backgrounds has been conducted (Tables 4.4., 4.5.). Again, the observed levels of the PAT protein were consistent for the respective whole plants, indicating that the *bar* gene is also stably expressed in different genetic backgrounds.

It is concluded that the *bar* gene present in transformation event CBH-351 will be expressed in all major tissues exposed to the herbicide in hybrid plants at a sufficiently high level to sustain tolerance during the growing season. Moreover, the *bar* gene is stably expressed within different genetic hybrid corn backgrounds.

Table 4.2. Mean (+/- Std, n=3) of Total Protein, Cry9C Protein and PAT Protein in plant tissues at 4 different Stages across the growing season, Johnston, IA, 1996.

	Stage 1			*Stage 2*			*Stage 3*			*Stage 4*		
	Protein mg/g	**Cry9C ug/g**	**PAT ug/g**	**Protein mg/g**	**Cry9C ug/g**	**PAT ug/g**	**Protein mg/g**	**Cry9C ug/g**	**PAT ug/g**	**Protein mg/g**	**Cry9C ug/g**	**PAT ug/g**
Whole Plant, Bt +												
Mean	12.5	250.0	189.7	7.8	230.3	105.7	5.7	96.1	44.4	2.9	21.8	6.8
Std	(4.6)	(78.6)	(23.5)	(1.9)	(5.5)	(16.7)	(1.4)	(26.9)	(5.4)	(1.6)	(1.1)	(1.6)
Whole Plant, Bt O												
Mean	17.3	bdl*	bdl									
Std	(0.7)											
Leaf, Bt +												
Mean	3.1	75.1	45.4	0.6	44.0	14.0	1.5	6.6	2.3	1.8	23.5	0.0
Std	(1.3)	(9.3)	(9.7)	(0.1)	(9.9)	(0.1)	(0.1)	(0.9)	(0.2)	(0.2)	(21.6)	(0.0)
Leaf, Bt O												
Mean	3.1	bdl	bdl	0.6	bdl	bdl	1.5	bdl	bdl	1.8	bdl	bdl
Std	(1.3)			(0.1)			(0.1)			(0.2)		
Root, Bt +												
Mean	0.7	55.7	39.1	0.3	25.8	4.4	0.3	4.6	0.6	0.6	10.5	2.2
Std	(0.1)	(4.1)	(2.7)	(0.2)	(19.2)	(0.8)	(0.2)	(0.4)	(0.5)	(0.2)	(7.4)	(2.8)
Root, Bt O												
Mean	(0.6)	bdl	bdl	(0.5)	bdl	bdl	(0.3)	bdl	bdl	(0.5)	bdl	bdl
Std	(0.2)			(0.2)			(0.1)			(0.2)		
Stalk, Bt +												
Mean				0.0	2.8	0.5	0.0	2.6	0.5	0.1	5.5	0.0
Std				(0.0)	(1.2)	(0.5)	(0.0)	(0.8)	(0.2)	(0.1)	(3.1)	(0.1)
Stalk, Bt O												
Mean				0.04	bdl	bdl	0.1	bdl	bdl	(0.4)	bdl	bdl
Std				(0.0)			(0.0)			(0.6)		
Tassel, Bt +									***Kernel, Bt +***			
Mean				6.9	175.0	4.2			Mean	6.7	18.6	17.8
Std				(0.1)	(100.4)	(1.2)			Std	(1.1)	(2.1)	(5.0)
Tassel, Bt O									**Kernel, Bt O**			
Mean				6.1	bdl	bdl			Mean	7.3	bdl	bdl
Std				(0.9)					Std	(1.0)		

* bdl = below detection limits

Data provided as the amount of protein per gram tissue on a dry weight basis.

Table 4.3. Mean (+/- Std, n=3) of Cry9C Protein and PAT Protein in plant tissues at 4 different Stages across the growing season, Johnston, IA, 1996.

	Stage 1		*Stage 2*		*Stage 3*		*Stage 4*	
	Cry9C %	**PAT %**	**Cry9C %**	**PAT %**	**Cry9C %**	**PAT %**	**Cry9C %**	**PAT %**
Whole Plant, Bt +								
Mean	2.10	1.62	3.07	1.40	1.72	0.83	0.91	0.26
Std	(0.70)	(0.44)	(0.66)	(0.32)	(0.42)	(0.27)	(0.55)	(0.09)
Whole Plant, Bt O								
Mean	bdl*	bdl						
Leaf, Bt +								
Mean	2.74	1.62	7.01	2.22	0.44	0.15	1.32	0.00
Std	(1.13)	(0.58)	(1.56)	(0.15)	(0.07)	(0.01)	(1.19)	(0.00)
Leaf, Bt O								
Mean	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Root, Bt +								
Mean	7.71	5.39	11.03	2.51	2.60	0.15	1.72	0.33
Std	(1.19)	(0.63)	(1.66)	(1.82)	(2.47)	(0.14)	(0.61)	(0.32)
Root, Bt O								
Mean	bdl	bdl	bdl	bdl	bdl	bdl	bdl	bdl
Stalk, Bt +								
Mean			14.00	1.50	9.72	2.00	5.07	0.08
Std			(7.07)	(0.71)	(1.06)	(0.47)	(0.80)	(0.00)
Stalk, Bt O								
Mean			bdl	bdl	bdl	bdl	bdl	bdl
Tassel, Bt +					***Kernel, Bt +***			
Mean			2.52	0.06	Mean		0.28	0.26
Std			(1.40)	(0.02)	Std		(0.02)	(0.01)
Tassel, Bt O					**Kernel, Bt O**			
Mean			bdl	bdl	Mean		bdl	bdl

* bdl = below detection limits

Data provided as % of total plant protein on a dry weight basis.

Table 4.4. Mean (+/- Std, n=3) of Total Protein, Cry9C Protein and PAT Protein in 4 different hybrids at 2 different Stages across the growing season, Johnston, IA 1996.

	Stage 1			*Stage 2*		
	Protein mg/g	**Cry9C ug/g**	**PAT ug/g**	**Protein mg/g**	**Cry9C ug/g**	**PAT ug/g**
Hybrid A - Bt +						
Mean	19.1	427.3	364.0	4.9	50.0	40.7
Std	(8.2)	(78.0)	(85.1)	(2.0)	(49.7)	(16.7)
Hybrid A - Bt O						
Mean	19.9	bdl*	bdl	8.0	bdl	bdl
Std	(1.1)			(2.0)		
Hybrid B - Bt +						
Mean	18.3	373.0	291.0	7.2	77.1	49.6
Std	(1.0)	(28.3)	(4.2)	(2.8)	(12.9)	(11.1)
Hybrid B - Bt O						
Mean	14.3	bdl	bdl	5.9	bdl	bdl
Std	(4.8)			(1.9)		
Hybrid C - Bt +						
Mean	11.6	422.7	227.0	6.8	100.2	60.9
Std	(6.3)	(53.2)	(33.4)	(1.3)	(24.9)	(9.5)
Hybrid C - Bt O						
Mean	14.4	bdl	bdl	7.1	bdl	bdl
Std	(3.0)			(1.3)		
Hybrid D - Bt +						
Mean	9.1	292.0	176.3	4.1	88.3	45.5
Std	(3.3)	(44.1)	(63.0)	(1.4)	(23.4)	(18.8)
Hybrid D - Bt O						
Mean	10.4	bdl	bdl	4.9	bdl	bdl
Std	(2.9)			(0.6)		

* bdl = below detection limit

Data provided as amount of protein on a dry weight basis.

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Table 4.5. Mean (+/- Std, n=3) of Cry9C Protein and PAT Protein in 4 different hybrids 2 different stages across the growing season, Johnston, IA, 1996.

	Stage 1		*Stage 2*	
	Cry9C %	PAT %	Cry9C %	PAT %
Hybrid A - Bt +				
Mean	2.61	2.09	1.42	0.97
Std	(1.10)	(0.52)	(0.58)	(0.03)
Hybrid A - Bt O				
Mean	bdl	bdl	bdl	bdl
Hybrid B - Bt +				
Mean	3.41	2.15	1.20	0.72
Std	(1.82)	(0.74)	(0.37)	(0.09)
Hybrid B - Bt O				
Mean	bdl	bdl	bdl	bdl
Hybrid C - Bt +				
Mean	4.70	2.31	1.49	0.93
Std	(2.45)	(0.76)	(0.27)	(0.20)
Hybrid C - Bt O				
Mean	bdl	bdl	bdl	bdl
Hybrid D - Bt +				
Mean	3.45	2.01	2.53	1.32
Std	(0.68)	(0.37)	(1.20)	(0.69)
Hybrid D - Bt O				
Mean	bdl	bdl	bdl	bdl

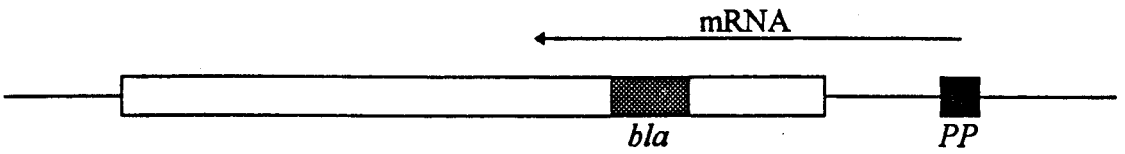
* bdl = below detection limits

Data provided as % of total protein on a dry weight basis.

Study No. 96QZM003

3. Cryptic gene expression

Southern and PCR analysis of the inserted DNA in event CBH-351 indicate that the bacterial *bla* gene sequence and the bacterial *ori*-containing sequence are present in transformation event CBH-351 (Annex 6). Although the *bla* gene is linked to bacterial transcription regulatory sequences and should not be transcribed in the plant, it is plausible that read-through events could result in the unexpected expression of the *bla* gene in event CBH-351 (e.g. when transcription starts at a plant promoter [*PP*] residing outside the inserted DNA; the transcribed mRNA could eventually span the *bla* gene present in the inserted DNA) (Figure 4.5.). Such an occurrence would be designated as "cryptic gene expression".



Inserted DNA

Figure 4.5. Example of how cryptic gene expression could possibly occur in CBH-351 (*PP* = Plant Promoter)

In order to determine whether unexpected expression of the *bla* gene in event CBH-351 is occurring, northern analysis of different tissues was performed using the *bla* gene as a probe. RNA transcripts comprising the *bla* gene sequences could not be detected in any of the tissues analyzed. (Annex 6).

It is concluded that the *bla* gene present in the inserted DNA in event CBH-351 is not expressed in the plant, by either direct gene expression or cryptic gene expression.

5. THE AGRONOMIC PERFORMANCE AND COMPOSITIONAL ANALYSIS

Transformation event CBH-351 has been field tested since 1994 in the United States, Canada, Belgium, France, Chile and Argentina. The United States tests have occurred under field release authorizations granted by APHIS (USDA Notification Numbers: 94-339-05n, 95-087-05n, 95-097-01n, 95-101-12n, 95-107-10n, 96-100-03n, 96-107-04n, 96-143-03n) and **CBI** *(95-089-04n, 95-094-03n, 95-261-07n, 95-272-08n, 95-284-13n, 95-320-02n, 96-059-02n, 96-075-04n, 96-086-07n, 96-094-16n, 96-100-05n, 96-102-02n, 96-108-09n, 96-115-05n, 96-115-09n).* Annex 8 includes termination reports submitted to the USDA for the environmental releases that have been completed in the United States.

A summary of the field results from the 1995 season was published this year in Jansens *et al.* (1997, see Annex 4). For the 1997 season, the CBH-351 event is being tested under an Experimental Use Permit, Number 70218-EUP-1. This EUP was approved by the Environmental Protection Agency on February 5, 1997 and covers expanded testing on up to 3,305 acres across 28 states/territories.

These trials included tests of the stability of insect control and herbicide tolerance, F_1 hybrid seed production trials, F_1 hybrid yield trials, efficacy trials on insect control and glufosinate ammonium tolerance and backcrossing programs with numerous elite inbred corn lines. In general, comparable transgenic and non-transgenic versions of the test material were grown for direct comparison. Observations were made by corn breeders and by other company researchers on agronomic performance and characteristics, and also on the disease and pest characteristics of the test material. Also, plant material was sampled for biochemical and molecular analyses.

In general, no differences could be identified between the transgenic (CBH-351, *Bt* Cry9C corn) and non-transgenic corn plants, with the exception of tolerance to glufosinate containing herbicides and resistance to lepidopteran larvae. From these comparative evaluations, it can be concluded that the transformation event CBH-351, *Bt* Cry9C corn, exhibits no plant pathogenic properties, is no more likely to become a weed than non-modified corn, and is unlikely to increase the weediness potential of any other cultivated plant or native wild species.

A. Event CBH-351 Confers Insect Protection

Event CBH-351 confers insect protection, or field efficacy, from ECB. Backcrossing programs for CBH-351 with inbred lines are in progress and the resulting inbreds will be used to produce commercial hybrids representing very diverse germplasm types. Protection from lepidopteran larval pests was observed in all of these genotypes. It can be concluded from the currently available data that neither the environment nor the hybrid genetic background in which event CBH-351 is present appear to have adverse effects on the stability and performance of the insect protection trait.

1. 1995 Field efficacy – PGS trials

Efficacy data was collected to evaluate the protection from ECB larvae under high artificial infestation conditions provided by CBH-351 (Annex 8, pg. 13-14, 50-51). Each corn plant in a row was artificially infested at mid-whorl and pollen shed stages with 500-750 neonate ECB larvae, to simulate first and second generation infestations of ECB, respectively. Visual evaluations were made for leaf damage at three weeks following the initial ECB infestation. Approximately eight weeks after the infestation with second generation ECB (just prior to harvest), the number of stalk tunnels and total stalk tunneling length for each corn plant were determined by splitting the stalks.

The results from the trials shown in Table 5.1. demonstrate the excellent ECB control that the Cry9C protein from corn event CBH-351 provides. CBH-351 plants were virtually free from ECB damage; at both the first (ECB1) and the second ECB (ECB2) damage evaluations (Jansens *et al.*, 1997, see Annex 4).

Table 5.1. Mean† ECB feeding damage expressed as mean (+/- standard deviation) on corn plants in Iowa and Belgium during 1995 season.

Entry	Leaf Damage Rating Guthrie Scale*	Number Stalk Tunnels/Plant	Total Stalk Tunnel Length/Plant (cm)
Iowa:			
CBH-351	0	0.06 (0.07)	0.09 (0.11)
Control	3	8.57 (2.9)	23.8 (4.3)
Belgium:			
CBH-351	0.61 (0.61)	0.10 (0.20)	0.14 (0.27)
Control	4.8 (0.75)	6.73 (0.8)	39.6 (11.9)

† Represents 3 replicates of 10 plants each

* Guthrie Scale (Mihm, 1983), 0=no leaf damage, 9=extensive leaf damage

To summarize, the corn plants expressing Cry9C insecticidal protein of event CBH-351 provided excellent season-long protection from ECB larvae, in these 1995 evaluations.

2. 1995 Field efficacy – Cooperator trials

Additional field evaluations were conducted in the USA with Bt Cry9C corn plants, event CBH-351, in cooperation with several different seed company partners, on multiple trial sites located across the corn belt (Annex 8, pg. 1-14, 22-23, 45-51). All trials utilized a randomized, complete block design. Each experiment was replicated two to four times. Standard corn cultivation procedures for the location were practiced. Comparisons were made between corn plants containing transformation event CBH-351 and non-transformed control plants with a similar genetic background. Efficacy data was collected to evaluate the protection from European corn borer larvae under natural and artificial infestation conditions.

The results of the field trials by cooperators demonstrated the excellent ECB control that the Cry9C protein from corn event CBH-351 provides. The bar chart shown below in Figure 5.1. graphically presents the results from five of these locations where significant ECB damage was observed. The CBH-351 plants were virtually free from ECB damage across five locations in the different genotypes tested. The comparable non-transgenic control plants had ECB damage ranging from 5.3 - 40.8cm of ECB tunneling/plant in the five locations.

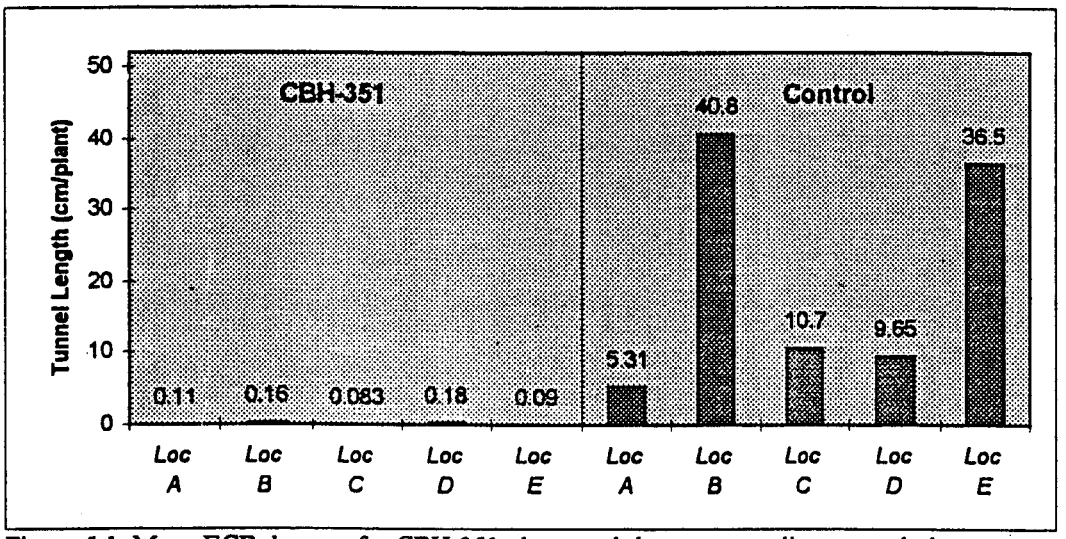


Figure 5.1. Mean ECB damage for CBH-351 plants and the corresponding control plants, expressed as centimeters of ECB tunneling/plant, for 5 different locations (Loc).

In summary, the results from cooperator trials are consistent with the results observed in the PGS field trials. All data indicate that event CBH-351 provides excellent protection in corn against the primary target pest, ECB.

3. 1996 Field efficacy

Field evaluations were conducted in the USA with *Bt* Cry9C corn plants, event CBH-351. The studies were carried out in cooperation with several different seed company partners, on multiple sites located across the corn belt (Annex 8, pg. 24-27, 29-32, 34). All trials utilized a randomized, complete block design. Each experiment was replicated two to four times. Standard corn cultivation procedures for the location were practiced. A number of comparisons were made: 1) efficacy across different environmental conditions for a given hybrid or group of hybrids, 2) efficacy across genotypes within a single environment, 3) efficacy of inbreds as compared to hybrids and 4) stability of efficacy across different generations of backcrossing. All trails compared corn plants containing transformation event CBH-351 with non-transformed control plants in a similar genetic background. Efficacy data was collected to evaluate the protection from ECB larvae under insecticide treatment, natural ECB infestation and artificial ECB infestation conditions. Efficacy across different environmental conditions for a given hybrid is presented in Table 5.2. Comparisons should be made for a single company, within each box, which shows the performance of a single hybrid across 2-3 locations.

Table 5.2. 1996 *Bt* Cry9C corn efficacy across environments for a given hybrid

Company	Hybrid	Site	CBH351		Isogenic Plant	
			# Tunnels	Length	# Tunnels	Length
Company A	Hybrid 1	Central IA	0.50	0.49	4.08	4.07
	Hybrid 1	Southern NE	0.00	0.22	12.50	12.46
	Hybrid 1	Western IN	0.40	0.18	4.87	14.80
	Hybrid 1	***Average***	***0.30***	***0.30***	***7.15***	***10.45***
Company A	Hybrid 2	Central IA	0.55	0.55	4.08	4.07
	Hybrid 2	Southern NE	0.00	0.00	12.50	12.46
	Hybrid 2	Western IN	0.60	0.26	4.87	14.80
	Hybrid 2	***Average***	***0.38***	***0.27***	***7.15***	***10.45***
Company A	Hybrid 3	Central IA	0.35	0.35	4.08	4.07
	Hybrid 3	Southern NE	0.00	0.22	12.50	12.46
	Hybrid 3	Western IN	0.50	1.86	4.87	14.80
	Hybrid 3	***Average***	***0.28***	***0.81***	***7.15***	***10.45***
Company B	Hybrid A	Northwest IL	0.00	0.00	1.15	4.24
	Hybrid A	Central IN	0.05	0.06	0.55	1.52
	Hybrid A	Eastern IA	0.02	0.05	1.45	4.80
	Hybrid A	***Average***	***0.02***	***0.04***	***1.19***	***4.01***
Company B	Hybrid B	Northwest IL	0.13	0.32	2.42	9.67
	Hybrid B	Central IN	0.00	0.00	0.52	1.73
	Hybrid B	Eastern IA	0.17	0.73	2.32	7.91
	Hybrid B	***Average***	***0.12***	***0.45***	***1.98***	***7.11***
Company B	Hybrid C	Northwest IL	0.33	0.99	2.16	7.46
	Hybrid C	Central IN	0.11	0.38	1.12	3.66
	Hybrid C	Eastern IA	0.22	0.81	1.98	6.32
	Hybrid C	***Average***	***0.23***	***0.77***	***1.85***	***6.07***
Company B	Hybrid D	Central IN	0.00	0.00	0.37	1.78
	Hybrid D	Eastern IA	0.02	0.04	1.25	4.41
	Hybrid D	***Average***	***0.01***	***0.03***	***0.94***	***3.50***
Company B	Hybrid E	Northwest IL	0.15	0.45	1.18	4.46
	Hybrid E	Central IN	0.05	0.13	0.27	1.27
	Hybrid E	Eastern IA	0.02	0.04	1.20	3.71
	Hybrid E	***Average***	***0.06***	***0.18***	***1.01***	***3.45***
Company C	Hybrid A1	Southern NE		0.34		28.75
	Hybrid A1	Central IA		0.00		23.65
	Hybrid A1	***Average***		***0.17***		***26.20***
Company C	Hybrid B2	Southern NE		0.20		31.60
	Hybrid B2	Central IA		0.07		26.95
	Hybrid B2	***Average***		***0.14***		***29.28***
Company A	B73CBH351.Mo17	Central IA	0.10	0.10	4.08	4.07
Company A	B73CBH351.Mo17	Southern NE	0.00	0.00	12.50	12.46
Company D	B73CBH351.Mo17	Northwest IL	0.14	0.35	1.83	5.08
Company E	B73CBH351.Mo17	Central IA	0.00	0.00	0.70	0.95
Company B	B73CBH351.Mo17	Eastern IA	0.00	0.00	2.83	8.71
	B73CBH351.Mo17	***Average***	***0.05***	***0.09***	***1.39***	***6.26***

Efficacy across genotypes within a single environment can be assessed by comparing different hybrids at each location in Table 5.3. A range of 3 to 9 different hybrids are compared per location. Different hybrids were tested in each company.

Table 5.3. 1996 *Bt* Cry9C corn efficacy across genotypes

Company	Hybrid	Site	CBH351		Isogenic Plant	
			# Tunnels	Length	# Tunnels	Length
Company A	hyb 1	Central IA	0.55	0.55	4.08	4.07
	hyb 2		0.50	0.49	4.08	4.07
	hyb 3		0.35	0.35	4.08	4.07
	CBH351/Mo17		0.10	0.10	4.08	4.07
Company A	hyb 1	Southern NE	0.00	0.00	12.50	12.46
	hyb 2		0.00	0.22	12.50	12.46
	hyb 3		0.00	0.22	12.50	12.46
	CBH351/Mo17		0.00	0.00	12.50	12.46
Company A	hyb 1	Western IN	0.60	0.26	4.87	14.80
	hyb 2		0.40	0.18	4.87	14.80
	hyb 3		0.50	1.86	4.87	14.80
Company F	CBH351, hyb 1	Central IA	0.58	1.91	3.67	12.28
	CBH351, hyb 2		0.14	0.36	3.22	9.88
	CBH351, hyb 3		0.17	0.42	3.38	10.94
	CBH351, hyb 4		0.11	0.28	2.88	8.73
	CBH351, hyb 5		0.50	1.27	5.10	16.76
	CBH351, hyb 6		0.22	0.56	2.25	6.03
	CBH351, hyb 7		0.19	0.48	3.11	8.75
	CBH351, hyb 8		0.13	0.32	1.31	3.71
	CBH351, hyb 9		0.13	0.32	1.77	6.84
Company B	CBH351 hyb 1	Northwest IL	0.00	0.00	1.15	4.24
	CBH351 hyb 3		0.13	0.32	2.42	9.67
	CBH351 hyb 4		0.33	0.99	2.16	7.46
	CBH351 hyb 6		0.15	0.45	1.18	4.46
Company B	CBH351 hyb 1	Central IN	0.05	0.06	0.55	1.52
	CBH351 hyb 3		0.00	0.00	0.52	1.73
	CBH351 hyb 4		0.11	0.38	1.12	3.66
	CBH351 hyb 5		0.00	0.00	0.37	1.78
	CBH351 hyb 6		0.05	0.13	0.27	1.27
Company B	B73(3)CBH351.Mo17	Eastern IA	0.00	0.00	2.83	8.71
	CBH351 hyb 1		0.02	0.05	1.45	4.80
	CBH351 hyb 2		0.30	1.00	1.32	4.29
	CBH351 hyb 3		0.17	0.73	2.32	7.91
	CBH351 hyb 4		0.22	0.81	1.98	6.32
	CBH351 hyb 5		0.02	0.04	1.25	4.41
	CBH351 hyb 6		0.02	0.04	1.20	3.71
Company C	CBH-351, hyb 1	Central IA		0.07		26.95
	CBH-351, hyb 2			0.00		23.65
Company C	CBH-351, hyb 1	Southern NE		0.20		31.60
	CBH-351, hyb 2			0.34		28.75

The efficacy for ECB observed for inbreds is given in Table 5.4. Where available, a corresponding hybrid is also included. At the bottom of Table 5.4, stability of efficacy across different generations of backcrossing is demonstrated. When appropriate, the number in parentheses indicates the backcross generation for the inbred.

Table 5.4. 1996 Bt Cry9C corn efficacy across generations - inbreds and hybrids

Company	Inbred	Site	CBH351		Isogenic Plant	
			# Tunnels	Length	# Tunnels	Length
Company G	Inbred G1	Central IL	0.33	1.10	2.83	12.47
Company D	CBH351 B73	Northwest IL	0.04	0.10	2.04	6.55
	B73CBH351.Mo17		0.14	0.35	1.83	5.08
Company E	B73	Central IA	0.05	0.05	1.90	3.07
	B73CBH351.Mo17		0.00	0.00	0.70	0.95
Company C	Inbred C1 BT	Central IA		0.08		13.13
	Inbred C5 BT			0.00		36.57
	Inbred C4 BT			0.00		41.23
Company B	B73(2)xCBH351	Eastern IA	0.09	0.22	4.58	17.02
	B73(4)xCBH351		0.02	0.06	4.58	17.02
	B73(3)CBH351.Mo17		0.00	0.00	2.83	8.71
Company B	B73(2)xCBH351	Eastern IA	0.09	0.22	4.58	17.02
	B73(4)xCBH351		0.02	0.06	4.58	17.02
	B73(3)CBH351.Mo17		0.00	0.00	2.83	8.71

In conclusion, the results of all of the field trials by PGS cooperators demonstrated that the transgenic Cry9C protein-containing corn event CBH-351 provides excellent ECB control. This insect protection is found across different environments and genotypes, for both inbreds and hybrids.

B. Glufosinate Ammonium Tolerance

Transgenic plants containing event CBH-351 were included in a tolerance screen with rates of glufosinate ammonium herbicide at 0, 600 and 1500 g. active ingredient/ha. Data was collected from field studies carried out in 1996 in Iowa. No detectable differences in plant appearance, such as leaf morphology and color, height, vigor, fertility, or flowering dates were observed for treated plants. *Bt* Cry9C corn, event CBH-351, exhibits tolerance to glufosinate ammonium at concentrations that provide effective weed control and excellent crop safety.

C. Agronomic Evaluations

1. General plant features

In field tests, no differences were observed between event CBH-351 and the non-transgenic counterpart or a non-transgenic standard line in seed germination, seedling emergence, leaf appearance, plant height, silk and anther color, tassel branching, cob color and size, plant vigor, flowering times, or seed set on hand-pollinated ears. For some CBH-351 inbred conversions, stunted corn plants have been observed. For most inbreds, ear quality and seed set appear to be adequate for hybrid seed production. Regardless of inbred appearance, hybrid seed produced from the inbreds gives plants with normal phenotype.

Volunteer corn plants were found the subsequent season, but the prevalence was not more than expected for standard corn plantings. Details can be found in the Termination Reports (Annex 8).

2. Disease and pest characteristics

Susceptibility to stalk-rotting diseases and ear molds (specifically, *Colletotrichum graminicola, Fusarium monillforms, Gibberella zeae,* and *Diplodia maydis*), leaf diseases (*Cercospora zeae-maydia, Helminthosporium* spp., *Erwinia Stewartii, Aspergillus flavus,* and *Puccinia sorghi*) and common smut (*Ustillago maydis*) along with general insect susceptibility were the same in the transgenic and non-transgenic plants with the exception of ECB tolerance, where a clear advantage was noted for the Bt Cry9C corn, event CBH-351, as noted in the termination reports (Annex 8). Likewise, tolerance to glufosinate-ammonium was observed with the transgenic event CBH-351 hybrids.

Therefore, agronomic evaluations in 1995 and 1996 have shown that the CBH-351 corn plants were indistinguishable from the control corn plants for general insect susceptibility (except ECB) and disease susceptibility, and weediness, throughout the season.

3. Effects on yield

Yield parameters, such as number of cobs per plant, cob weight, filling of cobs, or 1000 kernel weight, have been observed in a number of small-scale trials 1994 and 1995. No obvious differences between transgenic plants carrying event CBH-351 and non-transgenic plants were found.

Yield determinations for the 1996 season were made for five different hybrids in a single location in Iowa (Table 5.5). Data was collected to evaluate the yield under different insect treatment conditions. Moisture content and % stalk lodging (number of broken stalks ÷ total initial corn stand) data is included. One clear advantage of the CBH-351 corn event is the decrease in stalk lodging for the natural or artificially infested plots. ECB tunneling causes the corn plants to break, which increase stalk lodging and thus can have a significant effect on yield.

Table 5.5. 1996 *Bt* Cry9C corn yield under different insect treatment conditions - natural, artificial, insecticide

Entry	ECB Treatment	CBH351			Isogenic Check		
		Yield*	% H_2O	%SL†	Yield	% H_2O	%SL
Hybrid 1	Insecticide‡	152.0	24.2	4	145.0	21.8	14
	Natural	156.0	24.6	6	151.0	21.8	11
Hybrid 2	Insecticide	161.0	26.1	9	158.0	26.7	5
	Natural	142.0	25.6	3	160.0	23.8	12
	Artificial Infestation	165.0	26.8	3	147.0	26.8	16
Hybrid 3	Insecticide	164.0	27.2	8	162.0	27.0	3
	Natural	155.0	26.8	6	141.0	26.5	13
	Artificial Infestation	165.0	27.9	4	129.0	23.3	23
Hybrid 4	Insecticide	139.0	27.4	13	153.0	26.8	14
	Natural	145.0	29.0	8	154.0	26.1	11
	Artificial Infestation	147.0	29.4	8	142.0	24.3	24
Hybrid 5	Insecticide	156.0	32.0	4	150.0	33.0	11
	Natural	152.0	32.0	3	127.0	33.7	4
Avg of 5 pairs	Insecticide	154.4	27.4	7.6	153.6	27.0	9.4
Avg of 5 pairs	Natural	150.0	27.6	5.2	147.0	26.4	10.2
Avg of 3 pairs	Artificial Infestation	159.0	28.0	5	139.0	24.8	21

* Yield is expressed in bushels per acre.

† SL = Stalk lodging

‡ insecticide treatment was DiPel™

Plots treated with insecticide (DiPel™) were tested alongside plots with natural and artificial infestations of ECB larvae. The yields for CBH-351 were identical to yields in plots with insecticide treatment, with a 3 bushel/acre increase verses the natural or untreated control. As expected, the greatest yield enhancement for CBH-351 hybrids, 20 bushels/acre, was observed under the heavy artificial infestation conditions.

In conclusion, no decrease in yield parameters was observed across a wide range of environments and genotypes, for either inbreds or hybrids. Yield is an important agronomic indicator that is roughly the same or improved in the transgenic plants containing the CBH-351 event versus their non-transgenic counterparts, further evidence that this event displays no plant pest characteristics.

4. Composition profile of kernels

The composition profiles of kernels produced on insect resistant, herbicide tolerant hybrid corn plants of event CBH-351, of the kernels on their non-transgenic hybrid counterparts, and on kernels from other standard hybrids were determined. The seed samples were harvested from at least 5 Cry9C *Bt* plants, from 5 non-transgenic counterpart plants and 5 other standard non-transgenic hybrid plants. Five different hybrid backgrounds were included in the study, which was conducted in 1996 from corn grown in Iowa.

4.a. Proximate analysis

A list of the standard methods used for each test is included in Annex 5, pages 2-3. The results of the proximate analyses are presented below in Table 5.6. (additional information can be found in Annex 5). All data were statistically analyzed by a paired t-test. Hybrids with event CBH-351 expressing the Cry9C protein exhibited minor but significant differences for crude protein and crude fiber; all other traits were not statistically different than for the non-transgenic counterpart. Although values for all samples were slightly higher than the USDA-HNIS Reference for Protein and Ash, they were consistent across the hybrid backgrounds and are not of consequence.

Table 5.6. Composition of kernels (mean values of 5 individual samples)

% Content	Wet/Dry	Cry9C *Bt*[a]	Non-*Bt*	Standard	USDA -HNIS Reference[b]
Moisture	wet	9.51	9.42	9.46	8.03-12.71
	dry	n.a.	n.a.	n.a.	n.a.
Fat/Oil	wet	3.81	3.70	3.61	3.29-6.19
	dry	4.21	4.09	3.98	
Protein	wet	10.38	10.97	10.77	8.6-10.24
	dry	11.47*	12.11	11.89	
Fiber	wet	1.86	1.76	1.92	0.36-5.44
	dry	2.06*	1.94	2.12	
Ash	wet	1.7	1.65	1.76	0.98-1.44
	dry	1.87	1.82	1.94	

[a] Values in the Cry9C *Bt* column followed by an asterisk indicate a significant difference in the means (P=0.05) between the Cry9c *Bt* and the Non-*Bt* hybrids, according to a paired two-sample t-test.

[b] Range presented is a 95% confidence interval calculated from USDA-HNIS data (1989)

* Indicates statistical difference from the non-transgenic counterpart.

6. ENVIRONMENTAL IMPACT ASSESSMENT

In this section the expressed transgene products are considered for environmental impact; the Cry9C protein is considered first (Section 6.A.), followed by a consideration for the PAT protein (Section 6.B.). The proteins confer protection from lepidopteran larvae and glufosinate containing herbicides, respectively. Finally, the transformation event CBH-351 Bt Cry9C corn is reviewed (Section 6.C.) with respect to effects of such corn on non-target organisms and endangered species (Section 6.C.1. and 6.C.2.), environmental fate (Section 6.C.3.), weediness potential and outcrossing to wild relatives (Section 6.C.4. and 6.C.5.), and effects on current cultivation practices (Section 6.C.6.).

A. The Insecticidal Protein: *Bt* Cry9C

Bt -based products have been used for insect control for more than 30 years. *Bt* is a common spore-forming, gram-positive soil bacterium that contains crystal proteins that are insecticidally active. A long history of safe use is a primary reason that *Bt* proteins have been chosen as the basis for the first insecticidal plants produced by biotechnology. Although hundreds of *Bt* strains have been isolated, only 4 different strains have been commercialized to date, which is largely due to their high unit activity towards target pests. *Bt kurstaki*, a lepidopteran active strain, is the most widely used biological insecticide. *Bt tolworthi*, the strain from which the Cry9C protein was isolated has never been commercialized.

1. Mode-of-action of *Bt* proteins

The insecticidal mode-of-action of *Bt* proteins is a multi-step, complex, and not completely understood process. *Bt* mode-of-action can be divided into a series of critical steps: crystal solubilization, protoxin proteolysis, peritrophic membrane transport, brush border membrane binding, and pore formation. A concise description of the mode-of-action steps provides several pieces of evidence in support of the human safety and environmental safety properties that *Bt* proteins display.

The midgut environment of a lepidopteran larva is alkaline (pH>9.5) and probably reductive, which enables the *Bt* crystals to dissolve (Ogiwara *et al.*, 1992, Tojo and Aizawa, 1983). The protein crystals are stabilized by several disulfide bridges between the individual proteins within each crystal. Specific larval midgut proteolytic enzymes begin to cleave the solubilized inactive protoxin to a biologically active toxin form. Since mammals and other non-target pests (including most other insects) are unable to solubilize the *Bt* proteins, passage of the *Bt* proteins through their digestive systems occurs in the unaltered, and therefore non-toxic, form.

A family of putative insect midgut receptor binding sites has been described that bind *Bt* toxins irreversibly and with high affinity. Hoffman *et al.* (1988) were the first to illustrate toxin binding to isolated brush border membrane vesicles (BBMV) from the larvae of *Pieris brassicae* (cabbage butterfly). Since then, BBMV from many different insects have been studied with several different *Bt* toxin proteins (Ferré *et al.*, 1991, Garczynski *et al.*, 1991, Gould *et al.*, 1992,

Hofmann *et al.*, 1988, Hofmann *et al.*, 1988a, Knowles and Ellar, 1986, MacIntosh *et al.*, 1991, Van Rie *et al.*, 1989, Van Rie *et al.*, 1990, Van Rie *et al.*, 1990a, Wolfersberger, 1990).

Several research groups have examined the relationship of Cry protein binding by using competitive binding experiments (Van Rie *et al.*, 1989, Van Rie *et al.*, 1990, Wolfersberger, 1990). In some cases, the overlapping nature of the binding sites present in a single insect species appears to be complex. Regardless, the identification of specific receptor binding sites in the larval midgut of lepidopterans that are absent in other animals, including most other insects, gives additional support for the safety of *Bt* products.

At a some point, this binding can become irreversible, probably due to the insertion of at least part of the *Bt* proteins into the cell membrane. This insertion seems essential for the pore formation that leads to cell lysis, and establishes the correlation between irreversible binding and toxicity for the crystal proteins (Chen *et al.*, 1995). It is unknown whether the pores are entirely non-selective, or whether they have some selectivity for certain ions. Once pores are formed, the potassium pump stops functioning effectively and the columnar cells swell and lyse osmotically, leading to disruption of the integrity of the gut epithelium, starvation of the insect, and subsequent insect death.

2. Features of the Cry9C Protein

The Cry9C protein biochemistry is apparently similar to the more well known class of Cry1A proteins. Both classes display activity towards lepidopteran larvae through the mode-of-action steps as listed above: crystal and protoxin processing, receptor binding, membrane insertion and pore formation. The most striking difference for Cry9C versus the Cry1A family of proteins is a different spectrum of insect activity, which is apparently related to the unique receptor binding site found in the larval midgut as compared to Cry1A proteins.

2.a. Unique mode-of-action features

The Cry9C.PGS2 protein, used in the *Bt* corn CBH-351 event, has activity towards ECB, SWCB, Black cutworm (*Agrotis ipsilon*), and several Armyworm species (*Spodoptera*); the latter three are insects that can be difficult to control using the Cry1 and/or Cry2 proteins present in *Bt kurstaki* strains. An evaluation of the field efficacy of event CBH-351 against these pests is underway. For 1998, only ECB will be included on the EPA approved label.

The cleavage of the protoxin to an active form can also play a role in insect control specificity, as evidenced by the variety of proteolytic patterns observed in different insects (Bietlot *et al.*, 1990). For Cry9C from *Bt tolworthi*, the first 43 amino acids from the N-terminus are removed, along with the complete C-terminal half that follows the conserved block 5 region (Lambert *et al.*, 1996). For Cry1Ab, the first 29 amino acids of the N-terminal are cleaved, plus a large portion of the C-terminus (Höfte and Whiteley, 1989).

Heterologous competitive binding experiments were performed to determine the relationship between receptors for Cry9C and Cry1Ab in ECB and in resistant and Cry1Ab susceptible

colonies of Diamondback moth (DBM) (*Plutella xylostella,* (L.)) (Lambert *et al.*, 1996). For both of these insects, the receptors for Cry9C and Cry1Ab proteins were apparently distinct, with no overlap or competition. Of particular interest, both the resistant and the susceptible colonies of DBM demonstrated equivalent binding parameters for the Cry9C protein, while the binding of Cry1Ab was greatly reduced in the Cry1Ab resistant insect colony versus the susceptible control. These results are consistent with bioassay data observed for these colonies such that binding effectiveness was correlated with insect control. The fact that insects have different receptor sites for different *Bt* crystal proteins can be exploited for insecticide resistance management strategies (Roush, 1994)

2.b. Safety issues

The human safety of the Cry9C protein contained in the transformation event CBH-351 *Bt* Cry9C corn is being reviewed and evaluated by the EPA and FDA. Studies on acute mouse oral toxicity, *in vitro* digestibility and sequence homology to known allergens and toxins can be obtained from the EPA.

B. The Herbicide Tolerance Trait: Phosphinothricin acetyltransferase

A gene for phosphinothricin acetyltransferase (PAT), called the *bar* gene was isolated from *Streptomyces hygroscopicus*, a non-pathogenic bacterium. Members of the genus *Streptomyces* are gram-positive sporulating soil bacteria. In corn plants carrying event CBH-351, the *bar* gene that encodes the PAT protein is expressed and thereby produces PAT, which confers tolerance to glufosinate-ammonium herbicides.

1. Mode-of-action of PAT

Numerous compounds are synthesized by the genus *Streptomyces.* One is the antibiotic bialaphos, produced by both *S. viridochromogenes* and *S. hygroscopicus.* Bialaphos (syn. L-phosphinothricyl-L-alany-L-alanine) is an herbicidally active tripeptide consisting of two L-alanine molecules and an analog of L-glutamic acid called phosphinothricin. When it is released via peptidases, the L-PPT moiety is a potent inhibitor of gluamine synthetase (Bayer at el. 1972). L-PPT is the active component of the commercial herbicides, Herbiace® (Meiji Seika Ltd.) and Liberty® , Basta®, Ignite®, Rely® and Finale™ (AgrEvo GmbH). Herbiace® is bialaphos that is commercially produced using fermentation with *S. hygroscopicus.* The other herbicides mentioned are the ammonium salts of phosphinothricin, common name glufosinate-ammonia, and are chemically synthesized.

L-PPT is a potent inhibitor of the enzyme glutamine synthetase in both bacteria and plants, where it apparently binds competitively to the enzyme by displacing L-glutamate from the active site. Glutamine synthetase evidently binds L-PPT better than the natural substrate L-glutamate. Glutamine synthetase plays a central role in nitrogen metabolism of higher plants. It is the only enzyme in plants that can detoxify ammonia released by nitrate reduction, amino acid degradation

and photorespiration (Miflin and Lea, 1976). Ammonia, although a plant nutrient and metabolite, is toxic in excess and leads to death of plant cells (Tachibana et al., 1986).

Although the glutamine synthetase from both *S. viridochromogenes* and *S. hygroscopicus* are sensitive to L-PPT, the bacteria produce an inactivating enzyme for L-PPT, PAT. PAT catalyzes the conversion of L-PPT to N-acetyl-L-PPT in the presence of acetyl CoA as a co-substrate. N-acetyl-L-PPT does not bind to and inactivate glutamine synthetase, and thus, has no herbicidal activity. Therefore, plants expressing the PAT enzyme are resistant to the phosphinothricin class of herbicides. The PAT enzyme is encoded by the *bar* (*bial*aphos-*r*esistance) gene in *S. hygroscopicus*, and by the *pat* gene in *S. viridochromogenes*. These genes function both as an integral part of the biosynthetic pathway of bialaphos and as an enzyme which confers resistance (Kumada, 1986).

2. Safety Issues

The PAT protein has never been associated with any pathogenic reactions towards animals or human beings. The U.S. Food and Drug Administration has completed Food and Feed Safety Assessment review of several different corn lines genetically transformed to express a PAT protein. Data was provided to the FDA showing that "glufosinate tolerant corn is not materially different in composition, nutrition and safety from corn that is currently grown, marketed and consumed by animals and humans".

C. Transformation Event CBH-351, *Bt* Cry9C Glufosinate-ammonium Tolerant Corn

1. Effects on non-target organisms

Several different non-target organisms have been evaluated for their sensitivity to Cry9C protein as contained in corn plant powder and corn plant pollen from plants containing event CBH-351. No effects were detected during any of the studies which could be related to the presence of the insecticidal, Cry9C protein. Short summaries of these various studies are provided below. The EPA is evaluating these studies in a Registration Application submitted April 1997 (see Annex 9).

Collembola (*Folsomia candida*) were exposed for 28 days to bacterial and plant Cry9C powder by providing purified (bacterial) Cry9C protein or plant powder containing Cry9C protein mixed in a yeast food source. No effects were found that could be related to any of the Cry9C protein treatments. High doses of plant material (50% of diet) caused a small amount of mortality (14.3%) and reduced the total numbers of Collembola, but this reduction was found for both the Cry9C containing and for the non-transgenic control plant powders. Thus this result was likely due to the low nutritional quality of the plant powders.

Ladybird Beetle (*Hippodamia convergens*) insects were exposed to corn pollen from plants containing the CBH-351 event or to control corn pollen in a 21-day dietary toxicity test according

to the US guideline, OPPTS series 885-4350, under GLP conditions. No effects were observed with any of the treatments.

Cladoceran (*Daphnia magna*) were exposed to corn pollen from plants containing the CBH-351 event or to control corn pollen in a 48-hour static-renewal acute toxicity test, according to OECD guideline 202, performed under GLP conditions. No effects were observed with any of the treatments.

Earthworms were exposed to plant powder from plants containing the CBH-351 event or to control plant powder in a 14-day acute toxicity test according to OECD guideline 207, performed under GLP conditions. No effects were observed with the CBH-351 or control corn plant powder treatments.

Northern Bobwhite were exposed to diets of plant powder from plants containing the CBH-351 event or to control plant powder in a dietary feeding test for 5 days according to OECD guideline 205, performed under GLP conditions. No effects were observed with the diets made with the CBH-351 or control corn plant powder treatments.

A predatory non-target beneficial insect study was conducted in 1996 by entomologists from Iowa State University, using a randomized plot design with 3 replicates. No consistent pattern of differences in the number of predators trapped on the sticky traps was observed for any treatment (Bt +, Bt –, sprayed or unsprayed with Pounce™). Similar numbers of predators were observed on Bt + and Bt – corn plants during 5 minute observation periods. The most common predator observed and sampled on the sticky traps in this field study was *Coleomegilla maculata*.

A honey bee study is currently underway and preliminary data show no toxicological effects of the plant powder derived from plants containing event CBH-351. No Cry9C protein could be detected in catfish pellets made from *Bt* Cry9C corn. Therefore a waiver from performing a fish toxicological study is being sought from the EPA.

2. Effects on Endangered species

A list of endangered insect species was obtained from the Internet home page of the U.S. Fish and Wildlife Service, Division of Endangered Species. Dr. Marlin Rice, Professor of Entomology, Extension and Research at Iowa State University, reviewed this list and found that none of the listed species were pests of corn (Annex 7). Thus, *Bt* Cry9C corn does not pose any risk to any endangered insect species.

3. Environmental Fate

Plant powder prepared from corn plants of transformation event CBH-351, *Bt* Cry9C corn was mixed with soil and found to degrade over the 42 day period of the environmental fate study. The date of 'no effect' appears to be on and after day 30. Linear interpolation was used to estimate a

rough DT_{50} (time needed for a 50% reduction of insect activity) of 4.5 days. The median was 3.2 days. A median DT_{50} of 13.7 days was observed for DiPel™, a sprayable *Bt* formulation. This study was submitted to the EPA as part of the Registration Application in April 1997.

4. Weediness Potential

4.a. Corn

Corn is generally not regarded as a weed. It is frequently stated that corn is completely dependent upon humans for its survival. Indeed, the Union of Concerned Scientists (Rissler and Mellon, 1993) agree that contemporary corn is dependent on human intervention for survival and productivity. Corn is not listed as a noxious weed in the United States (USDA-AMS, 1994), nor is it listed as a weed anywhere else in the world (Holm et al., 1979). As is the case for many crop plants, corn does share some of Baker (1994) consensus list of characteristics common to many weeds.

4.b. Likelihood of Appearance of Glufosinate-resistant Weeds

The most likely way by which a weed could develop true resistance to glufosinate ammonium is through sexual transmission of the *bar* gene. This could occur where the crop and the related wild species are growing together and can exchange genetic material and produce fertile progeny. However, for corn in the United States, sexual transfer to weed relatives does not occur (see Section 2.B.).

Today there are large numbers of herbicide resistant weed biotypes, with over half of them resistant to triazines (Le Baron, 1991). Glufosinate ammonium is unrelated to triazines and has a different mode of action, i.e., it inhibits glutamine synthase. It is unlikely that weeds or any plant species will spontaneously develop resistance to glufosinate ammonium under selective pressure, because such a resistant plant must develop mutant forms of glutamine synthase that do not bind phosphinothricin and still recognize glutamic acid, and/or evolve a phosphinothricin detoxification system. Experimental work to create glufosinate ammonium resistant crop plants by selection has been ongoing for several years with no success.

In vitro mutagenesis studies in Dr. Howard Goodman's lab, Massachusetts General Hospital, demonstrated that glutamine synthase mutants that could no longer bind phosphinothricin could be obtained for the alfalfa glutamine synthase gene (personal communication, Günter Donn, AgrEvo GmbH). However, these mutants were very ineffective in using glutamic acid as a substrate. A plant bearing such a mutation would have difficulties surviving, because its ability to detoxify ammonia would be seriously decreased. This theoretical consideration is in accordance with the observations *in vitro* and in the field.

The introduction of resistance to the glufosinate ammonium herbicide has not caused *Bt* Cry9C event CBH-351 corn, to become a weed. Cry9C corn retains the same growth rate and growth habit as non-transgenic corn (Section 5.C.). It continues to be an annual which produces ears that

do not shatter and disperse their seed. In addition, *Bt* Cry9C corn is susceptible to ear rot disease and other disease and insect pests aside from target pests as its non-transgenic counterparts. Although event CBH-351 plants may produce volunteer plants the next season, the range in numbers of volunteer plants that occur is no different from the number expected for commercial corn production (Section 5.C.1.).

In conclusion, the likelihood of appearance of glufosinate-resistant weeds in the United States is extremely low.

5. Potential for Gene Transfer to Other Organisms

5.a. Outcrossing with wild species

As discussed in Section 2, hybridization between *Z. mays* and wild *Zea* species is theoretically possible. However, wild *Zea* species do not occur widely in the United States. Differences in factors such as flowering time, geographic separation, and developmental factors, for example, make crossing in nature in the United States only speculative. Crossing to the more distant relatives of *Z. mays* in the genus *Tripsicum* is very difficult and produces sterile offspring due to differences in chromosome number between *Zea* and *Tripsicum* species. Accordingly, there is little probability of unaided crosses between *Bt* Cry9C corn and wild relatives in the United States, and little potential for loss of biodiversity among wild relatives in the United States.

5.b. Outcrossing to cultivated corn

Wind pollination is the primary method of pollination in corn. However, outcrossing can be eliminated by several physical methods such as removal of the tassel and covering the silks with bags, or by geographic separation. These practices are practical for controlled crossings and the production of inbred corn. A high degree of self-pollination is ensured in the open-pollinated production of foundation and certified seed by planting well isolated blocks. The standard isolation distance for this production is 660 ft (approx. 200 m) from the nearest contaminating source (Wych, 1988). Controlled outcrossing or cross-pollination is the method by which two inbred lines are combined to produce hybrid seed. With hybrid seed production, as with foundation seed, fields must be isolated to prevent contamination. Hybrid seed is almost exclusively for commercial grain production in the US. Corn is open pollinated during commercial grain production.

When *Bt* Cry9C corn event CBH-351 hybrids are grown for commercial grain production, they will participate in unconfined outcrossing with other hybrid corn. Although event CBH-351 or its progeny from commercial grain production may arise as volunteers the following season, volunteer corn is generally removed. In Section 5.C.1. it is shown that Bt Cry9C corn is no more likely to produce volunteer plants than non-transgenic corn, and that volunteer plants can be eliminated by the application of herbicides, other than glufosinate ammonium, which are normally used for this purpose.

6. Effects of *Bt* Cry9C Corn on Current Farming Practices

6.a. Current farming practices - hybrids and herbicide use

Corn growers in the U.S. are currently served by more than 200 different seed companies, each of which produces and sells corn hybrids to farmers. Literally hundreds of different corn hybrids are sold across the U.S. Most corn growers plant a variety of corn hybrids on their farms, and often purchase seed from more than one seed company. The reason that several different hybrids are planted is to achieve a product mix which gives growers a range of maturities, pest tolerances, disease tolerances, stress tolerances, and yield potentials under different environmental conditions. Also, the specific hybrids that a grower plants is constantly changing. The average life of a corn hybrid is only 4-5 years, because within that period of time new corn hybrids are continuously being introduced that make the performance of the previous hybrids obsolete.

Nearly all of the corn acreage in the United States is treated with a herbicide. Products are applied preplant, pre-emergence and post-emergence to the corn crop. Herbicide programs in corn can vary due to the geographic area, weed spectrum, and first-year versus continuous corn. Farmers have traditionally relied upon triazine products in continuous corn where potential for carryover of the residual materials would not be a concern. Several weeds, however, have developed resistance to the triazines (LeBaron, 1991). Adverse weather conditions also reduce the effect of the triazines and other soil applied herbicides. In first year corn triazines are also widely used, however, their use is usually at lower rates and in combination with other soil applied products. Post-emergence applications of dicamba or 2,4-D are often used for broadleaf control. Recently, sulfonylurea herbicides have been introduced to control grass and broadleaf weeds post-emergence in corn. They are also used for problem weed escapes such as shattercane (*Sorghum bicolor*). In general, corn often receives a soil applied herbicide application and a follow-up post-emergence application. Due to potential crop injury, rotational concerns and weed competition, multiple herbicide applications applied post-emergence are not widely used in corn. Also, many products are used in combination as premixes or as tankmixes, to widen the spectrum of control. The reasons for this are to prevent corn injury, reduce weed pressure on the crop, and reduce rotational restrictions as to soybeans or other legumes. Harvest aid treatments of 2,4-D, dicamba, and other herbicide materials are sometimes used to facilitate harvest.

Problem weeds in corn include shattercane (*Sorghum bicolor*), johnsongrass (*Sorghum halepense*), quackgrass (*Agropyron repens*), fall panicum (*Panicum ciliatum*), foxtails (*Setaria* spp.), wild proso millet (*Panicum miliaceum*) and woolly cupgrass (*Eriochloa villosa*), as these are grassy weeds in a grass crop. Velvetleaf (*Abulilon theophrasti*), pigweeds (*Amaranthus* spp.), wild sunflower (*Helianthus annuus*), ragweeds (*Ambrosia* spp.) and smartweeds (*Polygonum* spp.) are broadleaf concerns. Perennial broadleaf species, such as hemp dogbane (*Apocynum cannabinum*), Canada thistle (*Cirsium arvense*), and dandelion (*Taraxacum officinale*) (weed problem in no-till), are difficult to control in corn. Perennials are difficult to control because they propagate by seed and/or underground plant parts. Control of these diverse species requires the use of multiple herbicide families and multiple applications.

6.b. Introduction of insect protection and herbicide resistance traits

The reasons for rotating crops and planting several different hybrids will not change with the introduction of *Bt* Cry9C corn hybrids. As insect protected and herbicide tolerant corn technologies becomes available, the traits will provide additional tools for the grower to improve productivity. However, the grower will still be faced with all of the other environmental and biological stresses to manage. The grower will continue to evaluate new hybrids in the search for higher yields. The addition of a *Bt* or *bar* genes will not alter the grower's desire to plant a number of different hybrids, to rotate fields from corn to other crops, and to plant new hybrid varieties with improved yield potential.

The expression of *Bt* proteins in corn plants, with activity that controls ECB, is a significant improvement over other current ECB control measures. By providing effective levels of the active protein at the proper time and in the proper crop tissues, the inherent problems associated with external insecticide applications can be overcome. More importantly, the *Bt* corn provides an environmentally safe control method that will provide benefits to a wider distribution of corn growers, by providing an economically feasible and effective control measure in fields where the current control measures are not feasible.

The use of glufosinate ammonium will have no effect on the normal growth patterns of *Bt* Cry9C corn, event CBH-351 plants. No effect on agronomic traits of event CBH-351 will be seen. Positive effects in corn cultivation will come from changes in tillage practices and herbicide use patterns. The broad spectrum, post-emergence activity of glufosinate ammonium will help increase the amount of conservation and no-till acres of corn planted in the United States. The use of glufosinate ammonium together with event CBH-351 will increase the adoption of post-emergence chemistry. Growers have the desire for a broad spectrum, post-emergent herbicide, as is evident in the adoption of post-emergence chemistry on other crops such as soybeans and wheat. Such a herbicide opportunity will give growers a way to move away from pre-emergence and residually active compounds.

Bt Cry9C event CBH-351 corn and glufosinate ammonium may positively impact current agronomic practices in corn by 1) offering superior ECB control, 2) preserving beneficial insects by reducing the use of broad spectrum insecticides, 3) offering a new insect resistance management tool for growers, 4) offering a broad spectrum, post-emergence weed control system; 5) providing the opportunity to continue to move away from pre-emergent and residually active compounds; 6) providing a new herbicidal mode of action that allows for improved weed resistance management in corn acreage; 7) offering the use of an environmentally sound and naturally occurring herbicide; 8) encouraging herbicide use on an as-needed basis; 9) decreasing cultivation needs; and 10) allowing the application of less total pounds of active insecticidal and herbicidal ingredients than presently used.

In particular, the availability of corn containing the *Bt* Cry9C protein will provide an even greater opportunity for the grower to manage risk, as compared to current options. The availability of corn containing a completely different *Bt* protein with a different binding site in the insect and a

different spectrum of insect control provides the grower with additional options beyond the current *Bt* Cry1A products. The *Bt* Cry9C technology will also be made available to a wide array of seed companies, making the product broadly accessible to corn growers through their preferred seed suppliers.

6.c. Insect resistance management

The successful use of new *Bt*-based biotechnology products will continue the development and implementation of sound management strategies. In the past, resistance management to chemical insecticides has occurred after resistance reached crisis proportions. In the few cases where resistance has developed to sprayable *Bt* products, it developed only after continuous and intensive use in tropical or sub-tropical environments with multi-generational insects. These cases provide lessons on how resistance can potentially develop, though the situation of how corn growers will use *Bt* corn for ECB control is a very different situation.

In general, a high dose-refugia management strategy will be utilized for *Bt* Cry9C corn. This strategy allows the maintenance of a susceptible insect population that could mate with any rare resistant insects that might evolve in the *Bt* corn. Offspring from this cross would all be controlled with the high dose of Cry9C protein found in transformation event CBH-351. The concentration of Cry9C protein in the plant tissues is about 2-3% of the total protein and thus is defined as a high dose (refer to Annex 4, pg 18). Growers will be instructed to keep some of their crop land planted with non-*Bt* corn. The amount of land set aside as a refuge will be determined by the informed grower in consultation with PGS and AgrEvo representatives, university and/or state extension researchers.

7. Statement Of Grounds Unfavorable

AgrEvo is unaware at this time of any conditions that are unfavorable to this request for nonregulated status of *Bt* Cry9C Corn, event CBH-351 hybrid corn seeds.

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September 13, 1994

Dr. Mark Williams and
Dr. Suri Sehgal
Plant Genetic Systems
Jozel Plateaustraat 22
B-9000 Gent, Belgium

Dear Sirs,

As a neutral expert on the relatives of corn in the U.S., I am glad to assist you.

The answers to your several questions are summarized here from several of my papers with copies enclosed for greater details about the relatives of corn from wild habitats in the U.S.

I. The genus Tripsacum is the second closest relative of corn. It is based on 18 pairs of chromosomes rather than the 10 pairs of corn and teosinte. All 16 species of Tripsacum are perennials. Only three of these are adapted to continental U.S. area. (T. dactyloides (2n & 4n), T. floridanum (2n), and T. lanceolatum (4n)).
Tripsacum dactyloides grows throughout the eastern half of the U.S. with the tetraploids near the east coast, sometimes in the salty soils where a river meets the ocean. The diploids are more in the mid-west of the U.S. Resistance to corn rootworm comes from T. dactyloides (Branson 1971).
T. floridanum is a small narrow-leafed diploid confined to open or the edge of pine lands in Florida.
It has been a source of genes for resistance to Helminthosporium turcicum (Hooker and Perkins, 1980) and corn leaf aphis (Branson, 1972).
T. lanceolatum is a tetraploid that occurs in the Southwest.

There is no evidence for natural hybridization between corn and Tripsacum in North America let alone just United States. There is controversial evidence for such introgression in South America.

Experimental hybridization and introgression between corn and Tripsacum is difficult but possible, usually requiring embryo culture. Some of the chromosomes have been cross-mapped by using old-fashioned marker genes (Galinat, 1973). Molecular markers are now being used for this purpose at CIMMTT in Mexico.

II. Teosinte is not only the closest relative of corn, it is the wild form of corn. It crosses freely with corn and is a good

source for corn improvement. Introgression from teosinte may be used to increase the hybrid productivity of corn and serve as a source of genes for disease and insect resistance. The perennial teosintes (2n & 4n) are a good source of resistance to virus diseases while the annual teosintes provide resistance to fungus diseases.

The habitat and distribution of teosinte is normally confined to Mexico, Guatemala and Honduras. It is know to have survived as an escape from cultivation in Florida and Texas. A day-neutral cultivar of teosinte occasionally grows as an escape in the corn breeder's nursery, but it is not considered as a serious weed and is easily killed with "Round-up" herbicide.

In parts of Mexico, teosinte is deliberately planted in corn fields because of its known beneficial effect on the corn. But the survival of the teosinte as a wild plant is damaged by introgression from corn and so natural selection in teosinte favors crossing barriers to corn such as different flowering times and gametophyte factors excluding corn pollen.

Details that I haven't reported here, you can find in my reprints enclosed.

If I can be of further assistance, do not hesitate to request it.

Sincerely,

Walton C. Galinat

Walton C. Galinat
Professor Emeritus

cc: Dr. Suri Sehgal
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United States Department of Agriculture
Agricultural Research Service
Southern Plains Area

ANNEX 2
Bt Cry9C Corn

September 26, 1994

Dr. Mark Williams
Research and Development
Plant Genetic Systems
Jozef Plateaustraat 22
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Dear Dr. Williams:

I congratulate you and your company on the development of new male-sterile system for maize. If transgenic plants can ever be readily be entered into the U.S. commercial market, this system should be well received.

Following are responses to your questions which should help in your patent process. I have provided a rather indepth review of the topic so that you can better address your patent concerns.

1) Three species of *Tripsacum* are native to North Amnerica. They are *Tripsacum floridanum*, *T. lanceolatum* and *T. dactyloides*. *T. floridanum* is found in S. Florida and is often used as a ornamental grass for landscaping yards. Other than that, *T. floridanum* is difficult to find. It is fairly rare and not extremely vigorous or aggressive. *T. lanceolatum* is found in S.W. Texas and S. Arizona. *T. dactyloides* is indigenous to most of the southern, central and northeast U.S. **No Tripsacum species cross readily with maize outside the laboratory**. Your research on statements found in the literature that *T. floridanum* crosses readily with corn applies only under laboratory, greenhouse or controlled field conditions. In fact, most *Tripsacum* species can cross readily with corn under laboratory conditions. To make such hybrids, pollen must be applied near the base of the maize silk. This requires husking back the maize ear and deliberately applying the *Tripsacum* pollen near the developing cob. **Tripsacum pollen tubes do not grow long enough to allow natural fertilization to occur between these species.** This is one barrier which prevents cross hybridization between the two species.

2) Hybrids made in the laboratory are either generated from small, nearly aborted hybrid seeds or from various embryo rescue techniques. If such seed were produced in nature, they would not survive. This goes for both maize x 2x (diploid) and 4x (tetraploid) *Tripsacum* crosses. The only known case of a naturally occurring "Zea"-*Tripsacum* hybrid is *Tripsacum andersoni*. It is native to Guatemala and is 100% male and nearly 99% female sterile. The very few seed this plant produces are products of apomixis. The plant itself has been propogated vegetatively by the indians for thousands of years. This cross is actually a 3x *Tripsacum* x maize hybrid where *Tripsacum* is the female parent. This cross is extremely difficult to make since *T. andersoni* is the only known representative of such a hybridization.

Very few *Tripsacum* x maize crosses have been successfully developed in any laboratory. I have not been able to generate one but several years ago, Harlan & deWet apparently developed a single hybrid which was completely male and female sterile.

3) All maize-*Tripsacum* hybrids are completely male sterile. Many are completely female sterile. About 10-20% of all maize-*Tripsacum* hybrids will set seed when backcrossed by maize. Some hybrids are vigorous, but none are able to withstand even the mildest winters and all eventually flower themselves to death. They can only be maintained indefinately in the greenhouse with human intervention. We have attempted backcrossing the hybrids with *Tripsacum* and have obtained some seed. However, generation of seed via this backcross pathway is even more difficult than a backcross by maize since it requires embryo rescue techniques. In addition, no one, even ourselves, has been able to take a maize-*Tripsacum* hybrid, backcross it by *Tripsacum* and successfully recreate a *Tripsacum* plant. We are trying this cross and the genetics just don't work in that direction. Once again, these are laboratory generated materials and as such could not be derived in nature.

4) *Tripsacum* in itself could be considered a non-aggressive weed. However, their is a growing demand for *Tripsacum* (eastern gamagrass) seed for planting. A few small companies in Nebraska, Missouri, Kansas and Oklahoma are commercially cultivating *Tripsacum* to be utilized as a new forage or haylage crop. We are in the middle of this research providing new genetic and germplasm materials. Sales are growing each year and the continued expansion of this market appears likely. Therefore, their population or levels of *Tripsacum dactyloides* found in the U.S. will be growing.

5) Crossability between all species of *Tripsacum* is excellent. This also goes for inter-ploidy crosses. This probably explains why we have no fewer than 11-16 taxonomic species of *Tripsacum*. Generally, I consider *Tripsacum* a germplasm swarm with multiple ploidy forms. If by some near miracle your gene jumps or escapes into *T. floridanum*, it is remotely possible that it could be eventually transferred into *T. dactyloides*. Again, I do not consider this a problem you should be concerned with.

6) I do not consider the probability of your genes escaping into *Tripsacum* a situation you should be concerned with. It is non-reality. However, in southern Florida, their does exist a sparsely dispersed, fairly rare, "native" teosinte called "Florida teosinte" . I say "native" with some hesitance since some believe it was introduced as a experimental forage crop many years ago and merely escaped. In any case, as with all teosintes, it can cross readily with maize. This occurs quite often in Central Mexico with *Zea diploperennis* and I suspect it could happen in Florida if their native teosinte were more widespread. You should check with the USDA in Florida to determine if they consider this species a true native. If so, this may present you with a minor problem considering the aggressive nature of anti-transgenic plant groups in the U.S. If they know their species, I suspect you will probably have to address the situation. As a geneticist, I do not foresee any problems.

To conclude, any concerns about your "transgenes" escaping into *Tripsacum* are not warranted. Their may however be some concern about their potential for escaping into the teosinte native to Florida. Good luck with your efforts. If you require any further assistance

with *Tripsacum* or the generation of haploid maize, let me know.

I also include a few manuscripts which may be relevant or of interest to you.

Sincerely,

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ANNEX 3
Bt Cry9C Corn

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A *Bacillus thuringiensis* Insecticidal Crystal Protein with a High Activity against Members of the Family Noctuidae

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The full characterization of a novel insecticidal crystal protein, named Cry9Ca1 according to the revised nomenclature for Cry proteins, from *Bacillus thuringiensis* serovar tolworthi is reported. The crystal protein has 1,157 amino acids and a molecular mass of 129.8 kDa. It has the typical features of the Lepidoptera-active crystal proteins such as five conserved sequence blocks. Also, it is truncated upon trypsin digestion to a toxic fragment of 68.7 kDa by removal of 43 amino acids at the N terminus and the complete C-terminal half after conserved sequence block 5. The 68.7-kDa fragment is further degraded to a nontoxic 55-kDa fragment. The crystal protein has a fairly broad spectrum of activity against lepidopteran insects, including members of the families Pyralidae, Plutellidae, Sphingidae, and Noctuidae. A 50% lethal concentration of less than 100 ng/cm² of diet agar was found for diamondback moth, European corn borer, ~~cotton bollworm~~, and beet armyworm. It is the first insecticidal crystal protein with activity against cutworms. No activity was observed against some beetles, such as Colorado potato beetle. The protein recognizes a receptor different from that recognized by Cry1Ab5 in *Ostrinia nubilalis* and *Plutella xylostella*. In *Spodoptera exigua* and *P. xylostella*, it binds to a receptor which is also recognized by Cry1Cax but with a lower affinity. In these insects, Cry1Cax probably binds with a higher affinity to an additional receptor which is not recognized by Cry9Ca1. Elimination of a trypsin cleavage site which is responsible for the degradation to a nontoxic fragment did result in protease resistance but not in increased toxicity against *O. nubilalis*.

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The major characteristic of the gram-positive, sporeforming bacterium *Bacillus thuringiensis* is the production of insecticidal crystal proteins (ICPs) during sporulation. To date, nearly 100 distinct crystal protein gene sequences have been published either in the general scientific literature (5, 8) or in patent applications. These crystal proteins have a specific toxic activity against certain lepidopteran, dipteran, or coleopteran larvae.

Several of the anti-Lepidoptera ICPs have a very high toxic activity against the early-instar larvae of agronomically important pest insects such as *Heliothis* and *Helicoverpa* spp. (budworms and bollworms), *Spodoptera* spp. (armyworms), *Ostrinia nubilalis* (European corn borer), and *Plutella xylostella* (diamondback moth) (12). However, some species of the family Noctuidae such as *Spodoptera frugiperda* and *Agrotis* spp. (cutworms) are insensitive to ICPs. Therefore, we have screened a subset of our collection of 12,000 *B. thuringiensis* isolates for activity against these members of the Noctuidae family. This screening program resulted in the discovery of a novel crystal protein with broad-spectrum activity against members of the Lepidoptera, including several species of the Noctuidae family and some ICP-resistant insects.

Here, we report the full characterization of this novel insecticidal crystal protein, including its gene and protein sequences, its homology with other crystal proteins, its activity spectrum, and the receptor-binding data. In addition, we describe the effect of elimination of a trypsin cleavage site on the activity of the crystal protein.

(The crystal protein, previously named Cry1H, was described in a patent application [9]. Preliminary findings were communicated at the Seventh International Conference on Bacillus [1993], Institut Pasteur, Paris, France, and the XXVIth Annual Meeting of the Society for Invertebrate Pathology, Asheville, N.C. [1993].)

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MATERIALS AND METHODS

Isolation of *B. thuringiensis* BTS02618A. *B. thuringiensis* BTS02618A was isolated as described previously by Travers et al. (11) by selective sample enrichment in a buffer containing sodium acetate followed by heat treatment and then plating out on agar plates. The BTS02618A strain reported here was isolated from grain dust collected in Cadlan, Bicol, The Philippines. The strain was deposited at the BCCM-LMG (Belgian Coordinated Collections of Microorganisms-Collection Laboratorium voor Microbiologie, R.U.G., Ghent, Belgium) under accession number P-12593.

Serotyping. Strain BTS02618A belongs to serovar H9 (tolworthi) as determined by J. F. Charles, Institut Pasteur, Paris, France.

ICP gene cloning and sequencing. Total DNA of BTS02618A was partially digested with *Sau*3A and size fractionated on a sucrose gradient. Fragments of DNA between 7 and 10 kb were ligated to *Bam*HI-digested and dephosphorylated pUC19 cloning vector (16). The recombinant plasmids were transformed into *Escherichia coli* MC1061 cells by electroporation. Transformed *E. coli* cells were grown on agar plates (containing 100 μg of ampicillin per ml), and about 10,000 clones were screened by Southern blot analysis with a mixture of nonradioactive digoxigenin-labeled *cry-1*-specific synthetic probes with the sequences 5'TTTTCATCCAGACAAAATT3', 5'TTCATCAAGATAGAATTCA3', 5'TTGGATTCGTATTAGTAAA3', 5'TCACATTTGTTTTAATCC3', 5'TCTATTGTCAATCGAATTT3', and 5'TTCTGTACTATTGATTGTA3'. Probe labeling and colony hybridization were carried out as described by the manufacturer (Boehringer Mannheim GmbH, Biochemica, Mannheim, Germany). A total of 31 positive clones were selected and analyzed by Southern blot analysis with *Alu*I, using the same set of probes to identify clones carrying the novel *cry* gene. One clone which carried a fragment with the full-length crystal protein gene was sequenced by the chemical modification method of Maxam and Gilbert (10).

Alignment of Cry9Ca1 with other crystal proteins. The amino acid sequence of Cry9Ca1 was deduced from the nucleotide sequence and aligned with the sequences of 43 crystal proteins (Table 1). The alignment was done with the Ifind program (IntelliGenetics, Mountain View, Calif.) with a window size of 10 amino acids and a word length of 2 residues. This program uses the Wilbur and Lipman similarity routine for the alignment. The percent amino acid identity was calculated by dividing the number of matches by the number of amino acid residues in the shorter of the two aligned sequences. This was done for the putative toxic

TABLE 1. Crystal proteins used for amino acid identity determination[a]

ICP	Accession no.	ICP	Accession no.	ICP	Accession no.	ICP	Accession no.	ICP	Accession no.
Cry1Aa1	M11250	Cry1Db1	Z22511	Cry2Ac1	X57252	Cry6Ba1	L07024	Cry12Aa1	L07027
Cry1Ab3	M15271	Cry1Ea1	X53985	Cry3Aa1	M22472	Cry7Aa1	M64478	Cry13Aa1	L07023
Cry1Ac1	M11068	Cry1Eb1	M73253	Cry3Ba1	X17123	Cry7Ab1	U04367	Cry15Aa1	M76442
Cry1Ad1	M73250	Cry1Fa1	M63897	Cry3Bb1	M89794	Cry8Aa1	U04364		
Cry1Ae1	M65252	Cry1Fa3	Z22512	Cry3Ca1	X59797	Cry8Ba1	U04365		
Cry1Ba1	X06711	Cry1Ga1	Z22510	Cry4Aa1	Y00423	Cry8Ca1	U04366		
Cry1Bb1	L32020	Cry1Ha1	Z22513	Cry4Ba1	X07423	Cry9Aa1	X58120		
Cry1Ca1	X07518	Cry1Ia1	X62821	Cry5Aa1	L07025	Cry9Ba1	X75019		
Cry1Cb1	M97880	Cry2Aa1	M31738	Cry5Ab1	L07026	Cry10Aa1	M12662		
Cry1Da1	X54160	Cry2Ab1	M23724	Cry6Aa1	L07022	Cry11Aa1	M31737		

[a] Crystal proteins are named according to the revised nomenclature (5).

fragments (from residue 1 to the C-terminal residue of the fifth conserved sequence block) and for the C-terminal fragments (from the first amino acid after the C terminus of the fifth conserved sequence block to the last amino acid). In cases when no typical conserved sequence block 5 was found (Cry2Aa1, Cry2Ab1, Cry2Ac1, Cry4Aa1, Cry6Aa1, Cry6Ba1, Cry9Ba1, and Cry11Aa1), the whole sequence was included in both alignments. In cases when no typical C-terminal half was found after the conserved sequence block 5 (Cry5A's, Cry3A1, Cry3Ba1, Cry3Bb1, Cry3Ca1, and Cry10Aa1), only the putative toxic fragments were included in the alignment.

Preparation and purification of crystal protein toxin fragment. A preculture was started by inoculating a 100-ml Erlenmeyer flask containing 10 ml of TB medium with a freshly grown colony of *E. coli* WK6(pHT-NSG1). This medium contained (per liter) 12 g of tryptone (Oxoid), 24 g of yeast extract (Oxoid), 6.3 ml of glycerol, 3.811 g of KH_2PO_4, 12.541 g of K_2HPO_4 (pH 7.1), and 100 μg of ampicillin per ml. Plasmid pHT-NSG1 was constructed by inserting the full *cry9Ca1* operon as an *Eco*RI fragment into the *Eco*RI site of pHT315 (1) behind the *lacz* promotor. The preculture was incubated in an Aqua-Shaker (Adolf Kühner AG, Birsfelden, Switzerland) at 28°C with shaking at 180 rpm for 5 h. The main culture was started by inoculating a 2-liter Erlenmeyer flask containing 200 ml of TB medium and ampicillin with 4 ml of preculture. This main culture was incubated at 25°C with shaking at 120 rpm. Gene expression was induced with isopropyl-β-D-thiogalactoside (IPTG) (final concentration, 1 mM) when the optical density at 600 nm exceeded 0.5 (after approximately 3 h). The induced culture was further grown for about 16 h. Before harvesting, the optical density of the culture was determined (by measuring the optical density of a 10-fold-diluted culture). This value was further used for determining the volumes of resuspension buffers to be added. Cells were harvested by centrifugation at 27,500 × *g* for 20 min, and the supernatant was decanted. The cells were washed by resuspension in 0.2 volume of phosphate-buffered saline (PBS; Oxoid, Columbia, Md.). The cells were pelleted by centrifugation at 27,500 × *g* for 45 min, and the pellet was resuspended in 1 ml of TES buffer (50 mM Tris · Cl, 50 mM EDTA, 15% sucrose [pH 8.0]) per 0.2 optical density at 600 nm unit. The suspension was frozen overnight at −20°C. After the suspension was thawed, 0.5 mg of lysozyme was added per 0.2 optical density unit. In addition, phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM to prevent proteolytic breakdown. The suspension was incubated at room temperature for 30 min, and cells were subsequently broken with a French cell press (SLM Instruments, Inc., Urbana, Ill.). Cellular debris was pelleted by centrifugation at 30,000 × *g* for 45 min. The following procedure was used to remove membrane proteins and lipids. The pellet was resuspended in TTN buffer (20 mM Tris · Cl, 1% Triton X-100, 1 M NaCl [pH 7.2]) and thoroughly mixed to wash the cells. The suspension was recentrifuged at 27,500 × *g* for 40 min. The washing step in TTN buffer was repeated twice. The resulting pellet was further washed twice with PBS-acetone (5:1, vol/vol) and finally washed once with PBS. The washed suspension was centrifuged, and the pellet was recovered. For extraction of the crystal protein, the pellet was suspended in 1 ml of alkaline buffer (50 mM Na_2CO_3, 10 mM dithiothreitol, 5 mM EDTA [pH 10.0]) per initial 0.2 optical density unit. Phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 mM. This mixture was shaken at 4°C for 2 h to solubilize the crystal protein inclusions. The solution was then centrifuged at 27,500 × *g* for 30 min, and the supernatant containing the protoxin was recovered. This protoxin solution was dialyzed against a buffer containing 20 mM Tris · Cl (pH 8.6) and 0.2 M NaCl by using a Spectrapor dialysis membrane (Spectrum Medical Industries Inc., Los Angeles, Calif.) with a cutoff value of 12,000 to 14,000. This material was used to prepare purified protoxin by ion-exchange chromatography.

The toxic fragment was generated by adding trypsin to the protoxin solution (1 mg/20 mg of protoxin) and incubating the mixture at 37°C for approximately 3 h. The degree of truncation was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. After completion of the truncation, the process was stopped by adding 0.1 mM phenylmethylsulfonyl fluoride. This crude toxin solution was redialyzed against a buffer containing 20 mM Tris · Cl (pH 8.6) (without salt). This solution was used for further purification of the toxin by ion-exchange chromatography. A sample of the crude toxin solution was applied to a MonoQ HR10/10 column (Pharmacia Biotech AB, Uppsala, Sweden). The material was eluted from the column in a linear gradient ranging from 0 to 0.6 M NaCl in 30 ml of 20 mM Tris · Cl (pH 8.6). Fractions containing the toxic fragment were identified by SDS-PAGE analysis and bioassays (see below). The toxin concentration was determined by optical density measurement at 280 nm and calculated on the basis of a molar extinction coefficient (ε_M) of 57,983 liter mol^{-1} cm^{-1}. The purified crystal protein solutions (protoxin or toxin) were stored at 4°C until use.

Determination of insecticidal activity spectrum. Toxicity assays were performed with neonatal larvae (except for *P. xylostella*, for which third-instar larvae were used) fed on an artificial diet. The diet was dispensed in Multiwell-24 plates (Corning Costar Corp., Cambridge, Mass.) or, for *Manduca sexta*, in 100-cm^2 square petri dishes (Bibby Sterilin Ltd., Stone, England). A 50-μl volume of each of five appropriate toxin dilutions was applied to the surface of the diet in each 2-cm^2 well (Multiwell-24 plates), or 200 μl was applied in each 4-cm^2 well (Sterilin plates). The plates were then dried in a flow hood. The dilutions were made in a PBS–0.1% bovine serum albumin (BSA) buffer. A total of 24 larvae (2 per well) were used per dilution except for *M. sexta* (20 larvae, 4 per 4-cm^2 well) and *Heliothis* and *Helicoverpa* spp. (20 larvae, 1 per well). The Multiwell plates were covered and placed at 25°C in 60 to 70% humidity with a 16-h-light, 8-h-dark cycle. Mortality was scored after 5 or 6 days, and 50% lethal concentration (LC_{50}) data were calculated by Probit analysis (6).

Receptor-binding experiments. Heterologous competition experiments were performed to study the relation between receptors for Cry9Ca1, Cry1Cax (named Bt15 in reference 14 but not deposited in any database and belonging to the Cry1Ca class of toxins), and Cry1Ab5 in *O. nubilalis*, *Spodoptera exigua*, a susceptible laboratory population of *P. xylostella*, and a Javelin-resistant population of *P. xylostella*. The resistant *P. xylostella* colony originated from Florida and was established from last-instar larvae and pupae collected from fields in Loxahatchee (10a). The colony was exposed to a Dipel crystal-spore preparation every three generations to maintain the resistance. The other insects came from our in-house colonies.

Biotinylation of the crystal protein was performed as follows. Toxin samples of the Cry9Ca1 Ala mutant (consisting of bands A and B [see below]) prepared as described above were first dialyzed against $NaHCO_3$ buffer (100 mM $NaHCO_3$, 150 mM NaCl [pH 9.0]). A 1-mg sample of Cry1Ab5, Cry1Cax, and Cry9Ca1 was mixed with 40, 120, and 280 μl of biotinyl-*N*-hydroxysuccinimide ester (BNHS; Amersham International, Little Chalfont, England), respectively. A higher ratio of label to toxin was used for Cry1Cax and Cry9Ca1 because labeling was less efficient. The mixture was incubated for 1 h at 4°C under constant agitation. Toxin samples were loaded onto a Sephadex G-25 (Pharmacia Biotech AB) column to separate biotinylated toxin from free BNHS. The A_{260} and A_{280} of all fractions were determined. Since free BNHS has a stronger A_{260} than A_{280}, it is possible to use this procedure to identify the fractions containing biotinylated toxin. The concentration of biotinylated toxin in these fractions was derived from the A_{280} readings. Fractions were not pooled. Biotinylated ICPs are referred to as B-ICP (e.g., B-Cry1Ab5).

Brush border membrane vesicles (BBMVs) from *O. nubilalis* and *S. exigua* were prepared as described by Van Rie et al. (14). BBMVs from *P. xylostella* were prepared from whole larvae as follows. Last-instar *P. xylostella* larvae were starved for 1 h and frozen in liquid nitrogen. A total of 20 ml of MET buffer [300 mM mannitol, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 17 mM Tris · Cl (pH 7.5)] was added to 900 frozen resistant larvae. A total of 30 ml of MET buffer was added to 1,500 larvae of the susceptible population. The respective mixtures were blended for two 1-min intervals at high speed with a blender (Waring Products Division, Dynamics Corp. of America, New Hartford, Conn.). One volume of 24 mM $MgCl_2$ was added, and the mixture was left on ice for 15 min. After centrifugation for 15 min at 6,000 × *g* and 4°C, the pellet carrying most of the cuticular fragments was

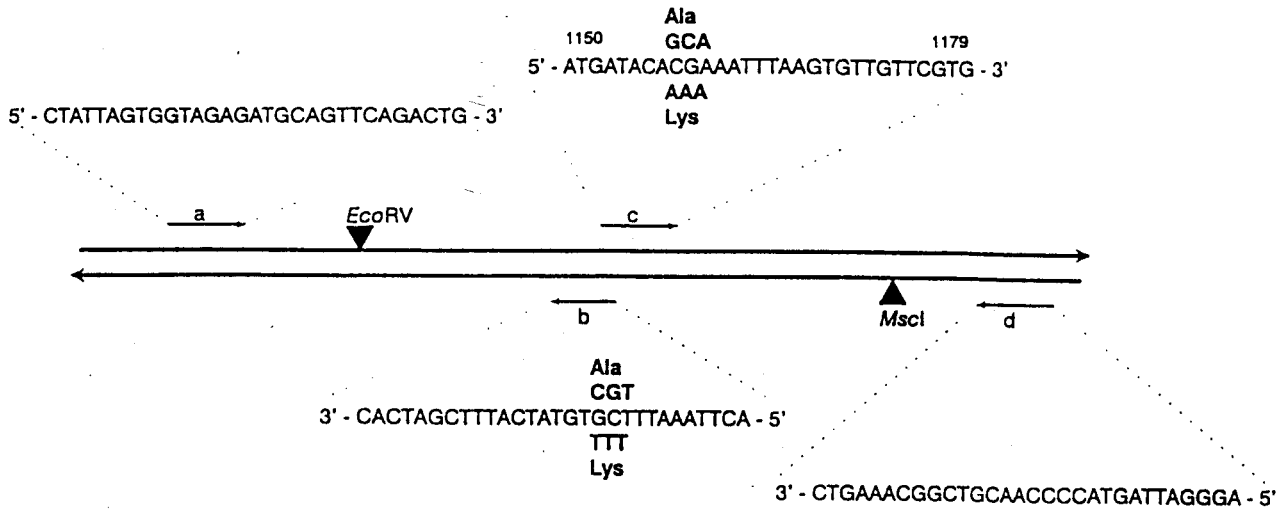

FIG. 1. Elimination of a proteolytic cleavage site by site-directed mutagenesis, using overlap extension. Plasmid pUC-NSG was used for these experiments. Two PCRs were carried out to generate two fragments, ab and cd, which incorporate the mutations. The lines with arrows represent the template DNA and primers in the 5'-to-3' direction. The sequences of the primers and the positions of the central primers on the full-length sequence are also shown. The mutant codons incorporated in primers b and c and the corresponding amino acids are given in boldface type above and below the original sequence. The triangles show the positions of the restriction sites used for further cloning of the fragments.

discarded. The supernatant was centrifuged at 11,000 × *g* for 30 min at 4°C. The resulting pellet had two layers. The bottom layer had a creamy color and probably contained mainly fatty acids. The upper, greenish layer was removed and resuspended in 0.5 homogenate volume of MET buffer. Another round of mixing, $MgCl_2$ addition, and centrifugation at 6,000 × *g* and 11,000 × *g* was performed, and the resulting pellet was resuspended in half-strength MET solution. Aliquots of this BBMV preparation were stored at −70°C until use. The concentrations of the BBMV preparations were determined by the method of Bradford (3).

Binding assays with B-ICPs on BBMVs were done as follows. An aliquot of BBMVs was thawed and centrifuged in a Biofuge (Heraus Christ GmbH, Osterode, Germany) at 13,000 × *g* at 4°C. The resulting pellet was resuspended in PBS–0.1% BSA to give a final concentration of 1 mg/ml. BBMVs were added to an appropriate amount of biotinylated ICP in PBS–0.1% BSA (in a total volume of 100 μl) and incubated at room temperature for 1 h. The mixture was centrifuged at 13,000 × *g* at 4°C for 10 min. The resulting pellet was washed with 500 μl of PBS–0.1% BSA and finally solubilized in 30 μl of sample buffer mix. Bound ICPs were visualized essentially as described by Bosch et al. (2). The solution was incubated at 95°C for 10 min. Samples were then separated by SDS-PAGE and subsequently blotted on a nitrocellulose membrane. This membrane was incubated overnight in blocking buffer, washed in TBST (20 mM Tris, 0.2% Tween 20 [Sigma, St. Louis, Mo.], 137 mM NaCl [pH 7.6]), and incubated for 30 min with streptavidin-horseradish peroxidase conjugate diluted 1/1,000 in Tris-buffered saline containing 0.1% BSA. The membrane was then washed for 10 min in TBST and for a few minutes in tap water. These washing steps were repeated three times. Excess fluid was removed from the washed membrane, and a mixture of equal volumes of detection solutions 1 and 2 (Amersham RPN 2109) was prepared and applied to the membrane. After a 1-min incubation period, the detection solution was drained off and the membrane was placed on a piece of filter paper (Whatman Int., Ltd., Maidstone, England) for 1 min to remove excess solution fluid. A scientific imaging film (Eastman Kodak Co., Rochester, N.Y.) was exposed for 30 s to the membrane wrapped in plastic foil. Additional exposures were taken after longer exposure times if needed.

Elimination of a proteolytic cleavage site. A trypsin cleavage site at residue 164 of the full-length crystal protein was removed by substituting the Arg residue with Ala. Substitution with Lys was also done because it is not always recognized as a trypsin recognition site (4) and because it is physicochemically (and possibly also functionally) equivalent to Arg. This was accomplished by site-directed mutagenesis via overlap extension by PCR as described by Ho et al. (7). The *cry9Ca1* operon was first cloned as an *Eco*RI fragment in the *Eco*RI site of pUC19, yielding pUC-NSG. A schematic diagram and the details of the procedure used are shown in Fig. 1. The positions of the primers and relevant restriction sites are also shown in Fig. 1. Both fragments were analyzed on an agarose gel containing 2% agarose in TAE buffer (40 mM Tris-acetate, 1 mM EDTA [pH 8.0]). The fragments (ab and cd) were eluted from the agarose gel with a Gene Clean kit (Bio 101, Inc., La Jolla, Calif.). A third reaction was then carried out with a mixture of the two fragments as the template and primers a and d as PCR primers. After the ad fragment was purified, it was digested sequentially with *Eco*RV and *Msc*I. This fragment was ligated to the *Eco*RV- and *Msc*I-digested pUC-NSG plasmid to replace the original fragment with the mutant fragment. The ligation mixture was electroporated into MC1061 cells, and several clones were analyzed by restriction analysis. Positive clones were sequenced to confirm the presence of the mutations. These were used for production of mutant Cry protoxins with Lys or Ala at residue 164.

Nucleotide sequence accession number. The *cry9Ca1* nucleotide sequence accession number at EMBL is Z37527.

RESULTS

Nucleotide and deduced amino acid sequences of the *cry9Ca1* operon. The full sequence of the *cry9Ca1* operon is presented in Fig. 2. The *cry9Ca1* operon consists of two open reading frames (ORFs) preceded by a promoter. The promoter sequence was identified by alignment with upstream sequences of other *cry* sequences and found to be identical to the *cry2Ac1* operon promoter (15). In addition, the protein encoded by the first ORF was identical to the protein encoded by *orf1* of the *cry2Ac1* operon. This protein is 167 amino acids long (or approximately 7 kDa in size). The second ORF encodes an insecticidal crystal protein of 1,157 amino acids (129.8 kDa in size). Putative ribosome-binding sites for both ORFs and an inverted repeat presumably functioning as a transcription terminator are also indicated.

Sequence similarities of Cry9Ca1 with other Cry proteins. The homology between the N-terminal half of Cry9Ca1 and corresponding fragments of the other crystal proteins was generally low and at best was 51% (for Cry1Ba1). Cry8Aa1, Cry1Bb1, and Cry5Aa1 showed homology of 49, 45, and 42%, respectively. All the others were less than 40% homologous. The level of homology of the C-terminal half and corresponding fragments of the other crystal proteins was generally higher. There was a striking 88% homology with Cry9Aa1. Three other crystal proteins, Cry8Aa1, Cry8Ba1, and Cry8Ca1, showed homology of 58, 54, and 55%, respectively.

Insecticidal activity spectrum. The Cry9Ca1 toxin showed a rather broad spectrum of activity against many agronomically important lepidopteran insects. The LC_{50} data of Cry9Ca1 toxin in comparison with the other most efficient ICPs on several lepidopteran insects are presented in Table 2. Above all, it is very toxic for members of the Noctuidae such as *S. exigua*, *S. littoralis*, *Mamestra brassicae*, and *Agrotis segetum*. Of all ICPs tested in our laboratory, it is the only one with activity

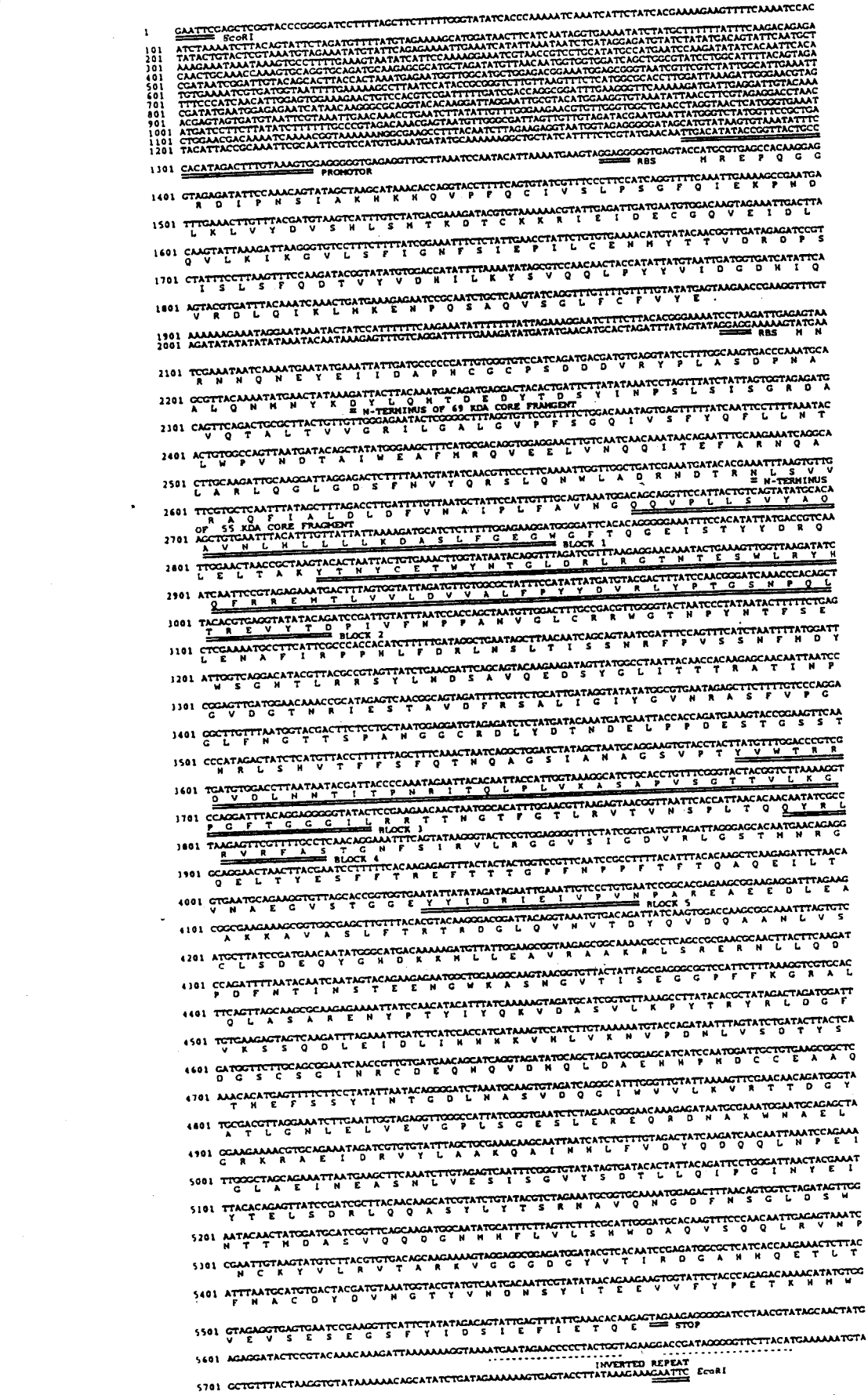

FIG. 2. Nucleotide and deduced amino acid sequence of the *crv9Ca1* operon. Underlined DNA and amino acid sequences indicate relevant features explained in the text. Further details are given in Results and Discussion.

TABLE 2. Insecticidal activity spectrum of Cry9Ca1 toxin

Insect species[a]	Larval stage	Cry9Ca1 ~~Cry1F~~ LC_{50}[b]	Fl_{95}min–max[c] (slope)	LC_{50}[b] of other most effective ICP[d]
Helicoverpa armigera	Neonate	>1,350.0		78.8 (Cry1Ab5)
Heliothis virescens	Neonate	51.8	39.2–64.9 (2.1)	1.6 (Cry1Ac1)
Mamestra brassicae	Neonate	78.8	53.5–111.4 (1.6)	22.0 (Cry1Ca1)
Manduca sexta	Neonate	83.0	66.9–103.4 (2.5)	5.4 (Cry1Da1)
Ostrinia nubilalis	Neonate	96.6	77.7–119.8 (2.5)	50.0 (Cry1Ab5)
Plutella xylostella^S	Third instar	6.5	4.9–8.2 (2.3)	1.2 (Cry1Ba1)
Plutella xylostella^R	Third instar	6.1	2.8–11.4 (1.5)	2.3 (Cry1Ba1)
Spodoptera exigua	Neonate	132.9	96.7–180.4 (2.7)	68.9 (Cry1Ca1)
Spodoptera littoralis	Neonate	65.5	44.9–93.7 (2.4)	155.0 (Cry1Ca1)
Spodoptera frugiperda	Neonate	>1,350.0		NT[e]
Agrotis segetum	Neonate	234.2	165.2–327.1 (1.6)	>1,350.0

[a] *Plutella xylostella*^{S} refers to a wild-type population of this insect, while *Plutella xylostella*^{R} is a population which has developed resistance to Cry1Aa, Cry1Ab, and Cry1Ac (10a).
[b] Results are expressed as nanograms of toxin per square centimeter of surface. See also Materials and Methods.
[c] Fl_{95}min–max, 95% confidence limit.
[d] The most effective ICP against the indicated insect as determined in our bioassays.
[e] NT, not tested.

against the last insect. It also shows a considerable activity against *Heliothis virescens* but not against *Helicoverpa armigera*, also members of the Noctuidae. The LC_{50} for *S. frugiperda* is more than 1,350 ng of toxin per cm^2 of diet agar surface. It is nearly as toxic as Cry1Ab5 for *O. nubilalis*, the most potent toxin so far described in the literature. In addition, it is highly toxic for both susceptible and Javelin-resistant diamondback moths.

Receptor-binding characteristics. All experiments were performed with Cry9Ca1 toxin samples consisting of mixtures of bands A and B (see below).

(i) *P. xylostella*. Heterologous competition experiments were performed to study the relation between receptors for Cry9Ca1, Cry1Cax, and Cry1Ab5 in *P. xylostella*. Whereas a 20-fold excess of unlabeled Cry9Ca1 could displace most of the bound labeled Cry9Ca1, only a 500-fold excess of unlabeled Cry1Ab5 was able to displace some of the bound labeled Cry9Ca1 in the susceptible population. These data indicate that these two proteins recognize different receptors. Whereas nearly all labeled Cry9Ca1 could be displaced by a 20-fold excess of unlabeled Cry9Ca1, only part of the labeled Cry9Ca1 could be displaced by a 20-fold excess of Cry1Cax. All labeled Cry9Ca1 was displaced by a 100-fold excess of Cry1Cax. This indicates that Cry1Cax recognizes the same receptor as Cry9Ca1, although with lower affinity. In the reverse experiment, unlabeled Cry9Ca1 could not compete with labeled Cry1Cax (Fig. 3). Such competition would be expected if the two proteins recognized the same binding site. We also compared Cry9Ca1 binding to BBMVs from a sensitive and a Javelin-resistant *P. xylostella* colony. The latter colony is highly resistant to Cry1Ab5 (10b) but is not resistant to Cry9Ca1 (Table 2). Binding experiments demonstrated that Cry1Ab5-binding ability is strongly reduced in the resistant strain. In contrast, Cry9Ca1 binds equally well to vesicles prepared from the sensitive strain and from the resistant strain. These data confirm the existence of two distinct receptors for Cry1Ab5 and Cry9Ca1, as inferred from the competition experiments.

(ii) *O. nubilalis*. A 1,000-fold excess of unlabeled Cry9Ca1 was not able to displace labeled Cry1Ab5. Unlabeled Cry1Ab5 shows only a weak competition for labeled Cry9Ca1. These data indicate that these two proteins recognize different binding sites.

(iii) *S. exigua*. Binding of labeled Cry9Ca1 can be prevented by a 20-fold excess of unlabeled Cry1Cax. Conversely, binding of labeled Cry1Cax could not be prevented by a 500-fold excess of unlabeled Cry9Ca1 (data not shown). Whereas a 20-fold excess of Cry1Cax could displace labeled Cry9Ca1, a 500-fold excess of Cry1Cax was needed to displace most labeled Cry1Cax.

Effect of the elimination of a trypsin cleavage site. Trypsin treatment of Cry9Ca1 protoxin showed that it is broken down initially to a fragment of 68.7 kDa (Fig. 4, band A), which is further processed (Fig. 4, lanes 2, 5, 8, and 11) to a 55-kDa fragment (Fig. 4, band C). Both fragments were purified and tested against *S. exigua*. The 68.7-kDa fragment was toxic at the concentrations tested, while the 55-kDa fragment was not

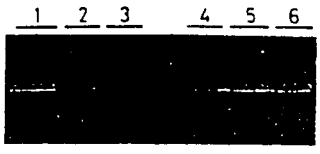

FIG. 3. Binding of biotinylated Cry1Cax on BBMVs of *P. xylostella* with a 20-, 50-, and 500-fold excess of Cry1Cax as a competitor (lanes 1, 2, and 3, respectively) or with a 20-, 50-, and 500-fold excess of Cry9Ca1 (lanes 4, 5, and 6, respectively).

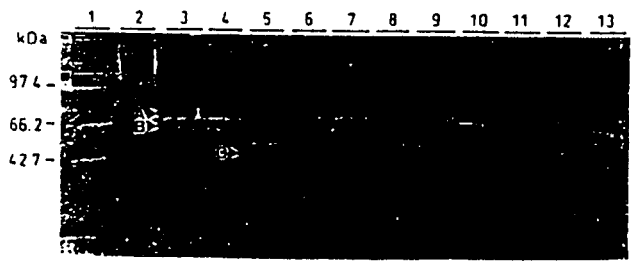

FIG. 4. Trypsinization of wild-type and mutant Cry9Ca1 crystal proteins and visualization by SDS-PAGE. Purified protoxin of the wild-type Cry9Ca1 (lane 2) and the partially digested and purified Lys and Ala mutants (lanes 3 and 4) were treated with 1 μg of trypsin per 20 μg of protein for 3 h (lanes 5 to 7), 20 h (lanes 8 to 10) and 24 h (lanes 11 to 13), respectively. Further batches of trypsin were added after 3 and 20 h. Samples were subjected to SDS-PAGE analysis. The N-terminal sequences of bands A, B, and C were determined and started at positions 44, 58, and 165, respectively, of the Cry9Ca1 protein sequence (see also Fig. 1). Lane 1 contains molecular mass markers.

TABLE 3. Comparative toxicity of wild-type and mutant Cry9Ca1 against *O. nubilalis*

Protein[a]	LC_{50}[b]	Fl_{95}min–max[c]	Slope
Cry9Ca1 protoxin	110.4	57.3–208.6	1.8
Cry9Ca1 (K) protoxin	41.3	33.9–49.8	3.5
Cry9Ca1 (A) protoxin	121.9	100.0–147.6	3.3
Cry9Ca1 (K) toxin	96.3	71.5–129.5	2.8
Cry9Ca1 (A) toxin	64.7	47.1–88.2	2.5

[a] (K) and (A) refer to the lysine and alanine mutants, respectively.
[b] Results are expressed as nanograms of toxin per square centimeter of surface.
[c] Fl_{95}min–max, 95% confidence limit.

toxic. We have attempted to prevent the breakdown of the protoxin or toxin to a nontoxic 55-kDa fragment during in vitro preparation, or possibly during passage through the insect gut, by elimination of the tryptic cleavage site at residues 164 to 165. This was done by substituting the Arg with the neutral amino acid Ala. It was also replaced with the physicochemically similar amino acid Lys.

Partially processed (67- to 69-kDa range) fragments of both mutant protoxins were purified (Fig. 4, lanes 3 and 4) and then tested for resistance to further proteolytic breakdown. It can be observed that only the Ala mutant is not broken down to the nontoxic 55-kDa fragment, even after 24 h of treatment and repeated addition of trypsin (Fig. 4, lanes 4, 7, 10, and 13). After 3 h of trypsin treatment, the protoxin (lane 5) and the Lys mutant (lane 6) are further degraded to fragments ranging from 65 to 50 kDa, with more of the 68.7-kDa fragment remaining in the Lys mutant. In later steps (lanes 8, 9, 11, and 12) the protoxin is more rapidly broken down than is the Lys mutant. Thus, the trypsin cleavage site is effectively eliminated by substitution of Arg at position 164 with Ala. In all samples including the partially digested Ala and Lys mutants (lanes 3 and 4), two major bands can be observed in the 65- to 69-kDa range, each consisting in fact of two bands. Both major bands were N-terminally sequenced. This revealed that the upper band (band A) consisted mainly of a fragment starting at position 44 (Asp) while the lower band (band B) consisted mainly of material starting at position 58 (Ile). N-terminal sequencing of the 55-kDa band indicated that this band starts at position 165. An additional band between the lower band and the 55-kDa fragment was not N terminally sequenced. Purified protoxin of the wild-type crystal protein (lane 2) and both mutants and partially digested and purified fragments of both mutants (lanes 3 and 4) containing the 68.7-kDa fragment (band A) and the 67.1-kDa fragment (band B) were tested against *O. nubilalis*. No significant differences between any of the five preparations were found (Table 3).

DISCUSSION

The novel crystal protein Cry9Ca1 described in this work is not just another crystal protein with a highly toxic activity against lepidopteran larvae. Indeed, its discriminative spectrum of activity makes it one of the most appealing ICPs for the control of agronomically important insect larvae either as sprays or through genetically engineered crop plants. Like some of the commonly used ICPs such as Cry1Ab5 and Cry1Ac1, it shows activity against members of the Pyralidae, the Plutellidae, the Sphingidae, and the Noctuidae (Table 2). Unlike Cry1Ab5 and Cry1Ac1, which are very toxic for *H. armigera* and *H. virescens*, it shows no activity against the former insect and a significant activity against the latter. In our bioassays, it was also very toxic for the diamondback moth, even for a Cry1Ab5-resistant population. Most striking, however, is its high activity against members of the Noctuidae. Like Cry1Ab5 and Cry1Ac1, it is very toxic for the European corn borer. Like Cry1Cax, it displays a strong activity against *S. exigua* and *S. littoralis* but not against *S. frugiperda*. Our bioassay data indicate that is the only ICP with activity against cutworms: it is toxic for the common cutworm (*A. segetum*), and preliminary experiments also indicate an activity against the black cutworm (*A. ipsilon*). In summary, the activity spectrum is most similar to that of Cry1Cax but it is also highly toxic for *H. virescens*, *O. nubilalis*, and *Agrotis* spp. In our hands, Cry1Cax is not toxic for either of these insects.

At the gene level, it is remarkable that the *cry9Ca1* gene is part of an operon. Cry2Aa2 and Cry2Ac1 are the only other examples of crystal protein genes which are organized in an operon (15). These operons contain the ORFs *orf1* and *orf2*, followed by the respective crystal protein genes. The *cry9Ca1* operon consists of two ORFs, the *cry9Ca1* gene and an ORF encoding a protein which is identical (except for one amino acid difference) to the *orf1* product of the *cry2Ac1* operon. The latter is 92% homologous to ORF1 of the *cry2Aa2* operon and appears to be missing 9 amino acids at the N-terminal region. No obvious function for ORF1 or ORF2 in Cry2 protoxin accumulation, solubilization, or toxicity of the crystal proteins was found by Wu et al. (15). We have not attempted to investigate the role of ORF1 in the *cry9Ca1* operon (e.g., by making deletion mutants). However, in analogy with the findings of Wu et al., it seems unlikely that ORF1 would play a crucial role in the activity of the Cry9Ca1 crystal protein.

Competition experiments have shown that Cry9Ca1 recognizes a receptor in *O. nubilalis* and *P. xylostella* different from that recognized by Cry1Ab5. For the latter insect, binding experiments with Cry1Ab5 and Cry9Ca1 on a resistant colony also strongly suggest the presence of two distinct receptors. Competition between Cry9Ca1 and Cry1Cax in both *S. exigua* and *P. xylostella* is somewhat more difficult to interpret. Whereas Cry1Cax can outcompete Cry9Ca1, the reverse could not be demonstrated. Perhaps Cry1Cax recognizes two binding sites, only one of which is also recognized by Cry9Ca1. Cry1Cax may bind preferentially to the receptor not recognized by Cry9Ca1. Competition experiments in which Cry1Cax was used as the competitor to displace labeled Cry1Cax and labeled Cry9Ca1 indicate that Cry1Cax has a higher affinity for the shared receptor than for the additional receptor not recognized by Cry9Ca1. Detailed radioligand-binding experiments would be needed to test this receptor model.

Upon in vitro trypsinization of the wild-type protoxin, toxic 68.7- and 67.1-kDa fragments were generated and were further processed to a nontoxic 55-kDa fragment after longer incubation. N-terminal sequencing of the 55-kDa fragment revealed the presence of a trypsin cleavage site (Arg at position 164). It was hypothesized that the same processing might occur in the larval insect gut by serine proteases, i.e., that when the protoxin was ingested, it would be quickly degraded to a nontoxic fragment. We reasoned that elimination of the trypsin cleavage site at position 164 by substitution with a neutral amino acid such as Ala or with Lys would result in an increased toxicity, since the end product of the proteolysis would be only a toxic fragment and hence a larger amount of toxic material could be expected to accumulate. In a similar experiment, Brinkmann et al. (4) successfully eliminated a protease cleavage target in *Pseudomonas* exotoxin A by replacement of the Arg residue with Lys. This resulted not only in protease resistance and full toxicity but also in a prolongation of the survival of the exotoxin molecule in mice. However, the Lys mutant of Cry9Ca1 was not resistant to trypsin. Probably not only the residue itself

but also the neighboring residues play a role in the recognition of a site by proteases. Trypsinization of the Ala mutant of Cry9Ca1 protoxin showed that it was resistant to proteolytic degradation (Fig. 4), but this did not result in increased toxicity against the European corn borer compared with that of the wild-type protoxin (Table 3). We did not analyze whether there is a significant difference in other insect species. Perhaps in *O. nubilalis* the time frame during which the intermediary protease-sensitive 68.7-kDa fragment is present (at certain concentrations) is sufficient to cause the killing of the larvae. Consequently, elimination of the trypsin site would not increase the activity. The situation might be different in other insect larvae. Indeed, in other poorly sensitive insect larvae (such as *S. frugiperda*), ingestion of a protease-resistant form of the toxin might result in increased toxicity because of the accumulation of the toxic fragment and the exceeding of a minimal threshold value. The Ala mutant of Cry9Ca1 is very valuable for the production of Cry9Ca1 toxin for use in bioassays, mode-of-action studies, etc., because a stable toxic fragment can be generated by simple trypsinization of the mutant protoxin. In fact, when we used the wild-type protoxin, it was difficult to control the processing in such a way that mainly 68.7- to 67.1-kDa material without the contamination of nontoxic 55-kDa fragment was generated. Moreover, because the 55-kDa and other fragments are physicochemically very similar they were very difficult to separate during purification.

In conclusion, Cry9Ca1 is a very valuable ICP because of its broad spectrum of activity and its specific receptor-binding characteristics. It might be of particular value for the control of some corn pest insects since it is toxic to the most important lepidopteran pest, *O. nubilalis*, and a number of secondary pest insects such as armyworms and cutworms. Bioassays on corn plants that have been engineered with Cry9Ca1 have shown that these plants are fully protected against the European corn borer and the common cutworm (unpublished results). Cry9Ca1 toxin binds to other receptors than the crystal proteins that are currently used in sprays or in engineered plants for the control of the Lepidoptera. Since the mechanism of resistance development against these toxins is primarily due to binding-site modification (13), Cry9Ca1 is a valuable tool for resistance management.

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Field Evaluation of Transgenic Corn Expressing a Cry9C Insecticidal Protein from *Bacillus thuringiensis* protected from European Corn Borer Damage

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Abbreviations: CaMV, cauliflower mosaic virus; ECB, European corn borer; ECB1 and ECB2, first- and second generation European corn borers, respectively; ELISA, enzyme-linked immunosorbent assay; LC_{50}, lethal concentration expected to cause 50% mortality.

ABSTRACT

The EUROPEAN CORN BORER, *Ostrinia nubilalis* (Hübner), is a devastating insect pest in the corn (*Zea mays* L.) growing regions of North America and Europe. Field evaluations in the USA and Belgium showed that transgenic corn events expressing Cry9C, an insecticidal crystal protein from *Bacillus thuringiensis* subsp. *tolworthi,* very effectively control both generations of the European corn borer. Second to fourth instar larvae fed with leaf material containing Cry9C of event CBH351 all died within four days. Cry9C events, expressing high levels of the insecticidal protein, showed minimal stalk tunneling after heavy artificial infestations. The event CBH351 showed in plots containing only Cry9C transgenic plants, 0.14 and 0.09 cm tunneling per stalk compared to more than 30 and 23 cm tunneling per stalk for the negative controls, in the Belgium and Iowa field trial, respectively. In plots containing 30% non-transgenic plants the event CBH351 showed only 1.45 cm tunneling per stalk. Leaf, tassel and pith tissue contained 39.0, 17.4 and 84.8 μg Cry9C protein mg^{-1} soluble protein, respectively, in analyses conducted at harvest of the Belgium trial. The implications of Cry9C use for resistance management strategies are discussed.

The EUROPEAN CORN BORER is a devastating insect pest in the corn growing regions of North America and Europe (Beck, 1987; Hudon et al., 1987). In the Corn Belt and southern France two generations per year are typical. The first ECB generation causes damage by whorl leaf and tassel feeding, and by stalk tunneling. Initially larvae feed on leaf tissue within the whorl. When the tassel starts to develop, second and third instar larvae move to the tassel and feed on the anthers. Fourth or fifth instar larvae enter the stalk and feed on the pith. The second generation ECB larvae initially feed on the exposed tassel, on accumulating pollen and on sheath-collar tissue. Later the larvae attack the cobs and bore into the shank and stalk. Yield losses are caused by reduced plant growth, by stalk lodging, ear droppage and by grain damage. Secondary infections by bacteria and fungi that occur as a result of the tunneling can also cause severe yield losses. ECB larvae on corn plants are difficult to control with chemical insecticides (e.g., organophosphates and synthetic pyretroids) because they are vulnerable to sprays or residues for only a short time before they bore into and are protected by the cob, sheath-collar or stalk. Proper timing of the chemical applications is crucial for success. Repeated applications of the chemical insecticides are often necessary.

The European corn borer is very susceptible to several *Bacillus thuringiensis* crystal proteins. Cry1Ab, Cry1Ac, Cry1B and Cry9C show high toxicity against ECB (MacIntosh et al., 1990; Denolf et al., 1993; Lambert et al., 1996). Corn engineered with insecticidal crystal protein gene(s) of *B. thuringiensis* appears to be a very effective strategy to protect corn against ECB. Corn events expressing Cry1A (Cry1Ab

or Cry1Ac) have been generated and showed high insect resistance in the field (Koziel et al., 1993; Amstrong et al., 1995). The Cry9C protein has a different activity spectrum than Cry1Ab or Cry1Ac proteins. For example, Cry9C is toxic to black cutworm (*Agrotis ipsilon* (Hufnagel)) larvae (Lambert et al., 1996). Also, Cry9C protein has shown to bind to a different midgut epithelium receptor than Cry1Ab in ECB larvae, a feature that could be exploited in insect resistance management (Lambert et al., 1996). Corn events expressing the Cry9C protein were generated and showed high ECB resistance in the lab (van Aarssen et al., in preparation). Here we report the field performance, at two locations, of selected Cry9C corn events under high artificial ECB infestation pressure. In the Belgium field trial, the effect of having non-transgenic plants present in the row of Cry9C expressing plants on the level of ECB resistance of Cry9C plants was studied. Cry9C protein levels were determined in different tissues during the growing season for a number of transformation events. High levels of Cry9C protein were produced and several events showed high European corn borer resistance. Implications of using Cry9C containing corn for Insect Resistance Management strategies are also discussed.

MATERIAL AND METHODS

Cry9C transgenic corn events contained a *cry9C* gene encoding a protein corresponding to the toxic fragment of the Cry9C protein (Lambert et al., 1996). CBH2.. events expressed the wild type, truncated Cry9C protein. In the Cry9C expressed by CBH3.. events, the arginine at position 164, susceptible to trypsin cleavage (Lambert et al., 1996), was replaced by a lysine residue (van Aarssen et al., in preparation). The *bar* gene encoding phosphinothricin acetyltransferase, providing resistance to glufosinate ammonium, was used as selectable marker gene (Thompson et al., 1987). Both the *cry9C* gene and the *bar* gene were under the control of the cauliflower mosaic virus 35S promoter (Harpster et al., 1988).

Belgium Field Trial

Thirteen entries, including ten entries having different hemizygous Cry9C events and three entries as negative controls, were planted at Plant Genetic Systems' experimental station in Astene, Belgium on May 22, 1995 (approval of the Belgian Authorities B/B/95/V16). The genetic background of the thirteen entries is given in Table 1. Standard maize production procedures for Belgium were practised. A strip-plot design with three replications was used to evaluate the ECB resistance of the ten Cry9C events and to study the effect of the presence or absence of non-transgenic plants among the Cry9C plants on the level of ECB resistance observed (Gomez and Gomez, 1984). The Cry9C events, the horizontal treatments were assigned to the horizontal-strip plots, and presence or absence of non-transgenic plants, the vertical treatments to the vertical-strip plots. Each intersection-plot (one experimental unit)

94 included ~25 plants, and was 3 m long, with a 1 m alley between plots. The *cry9C* and
95 *bar* genes were inherited as a single locus for all events, and all glufosinate-ammonium
96 resistant plants also carried a *cry9*C gene. A leaf paint assay with glufosinate-
97 ammonium was used to distinguish non-transgenic and Cry9C-transgenic for the
98 segregating hemizygous events. Forty-two days after planting, a formulation of 1%
99 Basta^R (200 g active ingredient L^{-1}) was painted onto two leaves and tolerance to the
100 herbicide was scored six days later. Plants showing no necrosis from the glufosinate-
101 ammonium treatment were considered to be Cry9C positive. Prior to ECB infestation,
102 in one vertical treatment all non-transgenic plants were removed, in the other non-
103 transgenic plants were not removed. Each intersection-plot contained ~10 Cry9C
104 plants. In the vertical-strip plots where the non-transgenic plants were not removed,
105 some non-transgenic plants perished because the glufosinate-ammonium painting
106 caused some plant mortality; therefore, the percentage of non-transgenic plants was
107 not always near 50% for the ten events (Table 1). The three negative controls were
108 randomized into the strip-plot design, but not considered in the strip-plot analysis of
109 variance . Note that for each of the three negative controls there were six replications.
110 The three replications adjacent to the Cry9C plots containing non-transgenic plants
111 and the three other replications adjacent to the Cry9C plots without the non-
112 transgenic plants were considered as two separate sets of data. To statistically
113 compare data of the ten Cry9C events and the negative controls, Cry9C plots
114 containing the non-transgenic plants and the adjacent negative control plots, and
115 Cry9C plots without the non-transgenic plants and the adjacent negative control plots

were considered as two different randomized complete block designs of thirteen entries with three replications.

All mid-whorl corn plants, except the H99 control plants, were three times infested on July 12, 14 and 19, 1995 with in total ~520 neonate first generation ECB larvae per plant. Neonate larvae were mixed with corn-cob grits and placed in the plant whorl with a mechanical larval dispenser (Barry and Chippendale, 1988). Plants were individually rated for ECB1 leaf feeding damage 3 wk after the final infestation using a modified Guthrie 0-9 scale (Mihm, 1983): 0 = no damage; 1 = pin holes (<0.1 cm); 2 = shotgun holes (<0.6 cm) on few leaves; 3 = shotgun holes (<0.6 cm) on several leaves; 4 = shotgun holes on several leaves or few lesions (0.6-2.5 cm); 5 = lesions on several leaves; 6 = long lesions (>2.5 cm) on several leaves; 7 = long lesions on 50% of leaves; 8 = long lesions on 70% of leaves; 9 = long lesions on most leaves. On August 11, 1995, when the plants were in the pollen-shedding stage, second generation ECB infestations were started on all ECB1 infested plants and also on the H99 control plants. During a 11 day period ~725 neonate ECB2 larvae, mixed with corn-cob grits, were placed in the axil of the cob and in the two axils above and under the cob of each plant. Approximately eight weeks after the last second generation infestation, ECB2 damage was evaluated by splitting the stalks and counting the number of tunnels and measuring the total tunnel length (in cm) per stalk. Plot means were calculated for all damage scoring. All data, except the leaf damage rating, were log(x+1) transformed before statistical analysis. Analysis of variance of the data was conducted using Agrobase/4 (Agronomix Software Inc., Manitoba, Canada). Because of low seed

availability there were some missing plots, for which the missing data formula technique described by Gomez and Gomez (1984) was used. Means were separated using least significant difference (LSD) at 0.05% level of probability.

Iowa Field Trial

Five Cry9C events and one negative control were planted in a randomized, complete block design with three replications at Slater, Iowa, USA on May 30, 1995 (acknowledgment by USDA of the notification for field release 95-107-10n). Standard maize production procedures were practised. The single-row plots were 3 m long, spaced 1 m apart, each row containing approximately 25 plants. The genetic backgrounds of the entries are given in Table 5. Twenty-nine days later all plants of the five events were painted with a glufosinate-ammonium formulation to identify Cry9C and non-transgenic plants. Non-transgenic plants were removed in all plots, so each plot contained ~10 Cry9C plants. Mid-whorl corn plants were infested with a total of 320 neonate ECB1 larvae on July 9 and 10, 1995. The same application technique was used as in Belgium. Plants were visually rated per plot for leaf feeding damage 1 wk after the final infestation using the modified Guthrie 0-9 scale. On August 11, 1995 plants in the pollen shedding stage were infested with 320 second generation larvae. Neonate ECB larvae, mixed with corn-cob grits were placed in the axil of the cob and in the two axils above and under the cob. On October 1 and 4, 1995, ECB2 damage was evaluated by splitting the stalks and measuring the number of tunnels and the total tunnel length in cm per stalk. Plot means were calculated for all damage scoring. The ECB2 data were log(x+1) transformed before statistical

160 analysis. Analysis of variance of the data was conducted using Agrobase/4 (Agronomix
161 Software Inc., Manitoba, Canada). Means were separated using least significant
162 difference (LSD) at 0.05% level of probability.

163 **Cry9C quantification**

164 During the growing season, Cry9C protein levels in different tissues of corn plants in
165 the field trial Belgium were quantified. On July 12, 1995 leaf material of mid whorl
166 stage plants was sampled. Fifteen days later, late whorl plants were harvested to
167 collect tassel primordia and leaf material. On August 7, 1995, tassel and leaf material
168 were taken from tasseling corn plants. When plants were silking and at harvest, on
169 August 18-25 and October 19-23, 1995, respectively, samples were taken from leaves,
170 tassels, silks, kernels and stalk. At silking, young kernels could no be easily
171 separated from the cob, so some cob material was included. At harvest the pithy
172 interior of parenchyma with vascular bundles (pith), and the outer shell of epidermis
173 and sclerenchyma of the stalk (cortex) were separately collected. Tissue samples were
174 frozen and crushed in liquid nitrogen. Soluble proteins were extracted in an extraction
175 buffer (50 mM Tris-Cl pH7.5, 5% glycerol, 100 mM KCl, 1 mM benzamidine.HCl, 5 mM
176 ε-amino-n-caproic acid, 10 mM EDTA, 10 mM EGTA, 1 $\mu g\ ml^{-1}$ leupeptin, 1$\mu g\ ml^{1}$
177 antipain, 14 mM beta-mercaptoethanol, 1 mM PMSF). After blending and
178 centrifugation the supernatant was used for protein quantification (Bio-Rad protein
179 assay; Bradford, 1976). Cry9C protein levels were determined in a double sandwich
180 ELISA (Clark et al., 1986), with immunoaffinity purified rabbit and goat polyclonal
181 antibodies specific for the Cry9C protein.

Toxicity bioassay of corn expressed Cry9C toxin

The insecticidal activity of the Cry9C protein, extracted from the pith and cortex of four CBH351 plants was tested in feeding bioassays with neonate ECB larvae. The dilutions of the plant extracts were based on its concentration of Cry9C protein, allowing direct comparison with a bacterial produced, trypsin-activated, purified Cry9C protein. The surface layer bioassay was described by Denolf et al. (1993). As a modification to the procedure, Multiwell-96 (Costar, Cambridge, MA) plates were used and one larva was placed in each well.

Leaf bioassays with first to fourth instar larvae

Leaf material from two control plants, four CBH351 plants and four CBH305 plants was randomly distributed in four Petri dishes and four neonate, second, third and fourth instar larvae of *O. nubilalis* larvae were added per dish. In total 16 larvae were used per larval instar. Dishes were sealed with parafilm. Every two days fresh material was added and mortality was scored. The greenhouse grown plants were in late whorl stage. The experiment was replicated three times.

RESULTS AND DISCUSSION

Belgium Field Trial

Ten independent events of transgenic corn expressing a truncated *cry9C* gene and three negative controls were evaluated in the field in Belgium. For the first generation infestations, whorl plants were infested with 520 neonate ECB larvae per plant. Pollen shedding plants were infested with 725 ECB larvae per plant for the second generation infestations. These numbers of larvae correspond with 26 and 36 egg masses, respectively. In the Corn Belt of the USA the acceptable standard threshold of natural infestation is 0.5 egg masses per plant in the corn field for the second generation, while in France it is less than one egg mass per plant (Labatte, 1991). The high artificial insect pressure on the field-tested plants resulted in considerable leaf damage and stalk damage in the negative control entries. Leaf damage rating was 4.8 to 5.72 for the control entries (Table 1) and total stalk tunnel length per plant was 30.7 to 49.2 cm (Table 2).

Table 1 shows that all Cry9C events had significantly lower ECB1 leaf damage than the control entries. CBH202, the less ECB1 resistant event had a leaf damage rating of 'few shotgun holes on few leaves' (~2), compared with a rating of 'lesions on several leaves' for the control entries (~5). 'Few shotgun holes' is considered as highly resistant (Guthrie, 1989). CBH352, CBH351, CBH301, CBH302 had a leaf damage rating of less than one or only 'pin hole' damage. Although there are significant differences between the events ($F=6.81$; $df=9,13$; $P=0.0003$; Table 1), differences of leaf feeding damage are small and all Cry9C events can be considered as highly ECB1

219 resistant. Cry1A type expressing maize events infested with 150 neonate larvae per
220 plant showed a leaf damage rating of less than four, most plants having a rating of
221 zero or one (Armstrong et al., 1995). Upon infestation with 1200 larvae per plant
222 Koziel et al. (1993) observed an average leaf damage rating for the Cry1Ab event 176
223 that corresponds to a fine 'window pane' and no 'pin hole' penetration.
224 The presence or absence of non-transgenic plants in the Cry9C plots had no significant
225 effect on the ECB1 leaf damage rating for the different Cry9C events ($F=1.29$; $df=1,13$;
226 $P=0.3735$). This is not the case for ECB2 stalk damage. The number of stalk tunnels
227 per plant and the total stalk tunnel length per plant (Table 2) were significantly
228 different ($F=47.13$; $df=1,13$; $P=0.0206$; $F=201.3$; $df=1,13$; $P=0.005$, respectively) for
229 Cry9C plots with or without non-transgenic plants. The higher number of stalk
230 tunnels per plant and the larger total stalk tunnel length per plant for plots containing
231 the non-transgenic plants may reflect an increased insect pressure. Since insect
232 movement is common for ECB larvae, larger instar larvae probably wandered from the
233 non-transgenic plants to the Cry9C plants.
234 In the plots where the non-transgenic plants were removed, all entries had
235 significantly less ECB2 damage than the control entries that showed with up to 42 cm
236 of tunnels per stalk (Table 2). The Cry9C entries could be divided in three
237 significantly different groups: CBH351, CBH352, CBH301, CBH302, CBH306 having
238 less than 0.25 tunnels and less than 0.25 cm total tunnel length per plant (Table 2,
239 Figure 1). CBH351, CBH352, CBH301 and CBH302 showed also virtually no shank
240 and cob feeding (results not shown). A second group, CBH202, CBH353, CBH201 and

CBH203B had a low ECB2 resistance: 2.83-3.40 tunnels and 13.0-16.3 cm tunnels per plant. CBH305 falls between with 0.76 tunnels and 2.19 cm tunnels per plant. Cry1Ab events 171 and 176 generated by Koziel et al. (1993) had a mean tunnel length of 4.97 and 3.0 cm, respectively, with 1200 ECB2 larvae giving an average of 57.4 cm tunneling in their controls. Armstrong et al. (1995) showed that the 34 highest ECB2 resistant Cry1A type events had 0-2.5 cm tunneling in the 1993 field trial. An ECB2 infestation of 300 larvae per plant gave a mean of 45.7 cm tunneling in their control plots.

In the plots where non-transgenic plants were not removed, events CBH351, CBH352, CBH301 and CBH302 were again highly resistant and had significantly less ECB2 stalk damage than the six other Cry9C events (Table 2). The number of stalk tunnels and total stalk tunnel length for these two groups were 0.80-1.47 tunnels and 1.45-2.97 cm tunnels per plant and, 3.77-5.50 tunnels and 12.8-29.3 cm tunnels per plant, respectively. Importantly CBH306 and to a lesser extent CBH305 were highly resistant only when non-transgenic plants were removed, i.e. 0.07 cm and 2.19 cm tunneling per plant. Instead in the plots with non-transgenic plants, the events showed 14.4 and 12.8 cm tunneling, respectively, which cannot be considered as highly resistant. Probably these two events are not fully stalk resistant to larger ECB instar larvae that frequently migrate from the non-transgenic plants onto the transgenic plants. Ross and Ostlie (1990) found that 50% of hatching ECB larvae placed on a corn plant left this plant, mainly within the first 48h. The radius encompassing 90% of the recovered larvae included the infested plant and two plants on either side within the

same row. Gould (1994) made a same observation with 50% of the neonate ECB larvae leaving the corn plant within 36h. Furthermore 90% of the larvae left the transgenic Cry1Ab corn plant. In one of the resistance management strategies (McGaughey and Whalon, 1992), it is proposed to reduce selection pressure on the insect populations by mixing resistant and susceptible seeds for planting. A main prerequisite for this strategy to work is a minimal migration of larvae between plants (Mallet and Porter, 1992) and/or excellent control of late instar ECB larvae.

Iowa Field Trial

Five Cry9C events tested in Belgium were also field tested at Slater, Iowa. Levels of artificial infestations were lower than in Belgium. However, the natural ECB density in the field was very high. At harvest, this resulted in 23.8 cm total tunnel length per plant for the B73 negative control (Table 3). In contrast, CBH351 showed a very high ECB2 resistance with only 0.06 tunnels and 0.09 cm tunneling per stalk (Table 3). Total length of stalk tunneling for CBH202, CBH203B and CBH353, 14.7-18.9 cm per stalk, was not significantly different from the B73 control. CBH305 showed intermediate stalk damage, 6.33 cm per stalk, significantly less than B73 but more than CBH351. Results at the Belgium and Iowa locations were very similar for the five tested Cry9C events.

Cry9C quantification

Cry9C protein was quantified for several events in different tissues over the growing season. Cry9C protein accumulation in leaf material of CBH301 and CBH351 was high, events which demonstrated excellent protection from ECB. The expression in

leaves of CBH301 decreased from 19.0 to 3.05 μg Cry9C mg^{-1} soluble protein over the period that the plants were sampled (Table 4). CBH351 had an expression level fluctuating between 24.3 and 39.0 μg Cry9C mg^{-1} soluble protein. The highest level observed of expression of CBH351 was about 10-fold higher than the highest level of four μg mg^{-1} soluble protein found in transgenic Cry1Ab corn events driven by the CaMV 35S, PEPC promoter, or pollen specific promoter (Koziel et al., 1993), and in transgenic Cry1A type corn events driven by the CaMV 35S promoter (Armstrong et al., 1995).

CBH202, CBH203B and CBH353 expressed over the growing season 0.76-0.22, 0.20-0.05 and 3.08-0.07 μg Cry9C mg^{-1} soluble protein in the leaves, respectively (Table 4). These lower levels correlated with their low ECB2 stalk resistance (Table 2), however levels were sufficient to give good ECB1 resistance (Table 1). The expression levels of CBH305 were about one μg Cry9C mg^{-1} in mid and late whorl plants and around 0.5 μg Cry9C mg^{-1} on the three sampling dates later in the season. On the three first sampling dates CBH306 had more than two μg Cry9C mg^{-1} soluble protein in their leaves, on the fourth date it had reduced to 0.32 μg Cry9C mg^{-1} soluble protein and at harvest it was 1.63 μg Cry9C mg^{-1} soluble protein (Table 4). On average expression levels of CBH306 were higher than those of CBH305. Expression level of the CBH306, and to a lesser extent CBH305, is intermediate, which is in correspondence with ECB2 stalk resistance of CBH306 and CBH305 in the Belgium field trial. Cry9C protein was also quantified in other plant tissues for CBH351 and CBH203. Expression of Cry9C in tassel, silks, kernels and stalk was low for the event CBH203

(Table 5). As was observed for leaves, levels of Cry9C protein in the tassel of CBH351 were very high : i.e. 11.7-24.0 µg Cry9C mg^{-1} soluble protein. Importantly expression remained high over the growing season. Results for kernels and silks of CBH351 showed a high, but decreasing expression level (Table 5). The highest expression level was found in the stalk of event CBH351 (Table 5). In silking plants 47.5 µg Cry9C mg^{-1} soluble protein was found and expression was even higher two months later at harvest, with 84.8 µg Cry9C mg^{-1} soluble protein in the pith and 74.2 µg Cry9C mg^{-1} soluble protein in epidermis and sclerenchyma of the stalk (cortex).

To confirm these high levels of Cry9C in the stalks of event CBH351, proteins were extracted from pith and cortex of four field grown plants. The Cry9C protein level in the extracts was determined by ELISA and the toxicity of the plant produced Cry9C protein was compared with bacterial Cry9C toxin. The LC_{50} values of the eight extracts were not significantly different from the LC_{50} values of bacterial Cry9C toxin, except that for the extract of the cortex of plant 29a there was no overlap of the 95% confidence limits (Table 6). Because the LC_{50} values of the extracts are not different from the expected LC_{50} value, we can conclude that the ELISA values are correctly measured and that all Cry9C protein present in the CBH351 plants is active. Koziel et al. (1993) found 1.7 µg Cry1Ab mg^{-1} soluble protein in the pith for the CaMV 35S driven event 171 and 0.075 µg Cry1Ab mg^{-1} soluble protein for the PEPC/pollen driven event 176.

If Cry9C protein is expressed on a per gram fresh weight basis the relative Cry9C concentrations in different tissues changes. For CBH351, at harvest the

329 concentrations of Cry9C protein were 39.0, 17.4, 6.8, 1.8, 84.8 and 74.2 μg mg^{-1} soluble
330 protein, compared with 225, 94, 44, 12, 18 and 23 μg g^{-1} fresh weight for leaf, tassel,
331 silks, kernels, stalk pith and stalk cortex, respectively. This is due to a low amount of
332 extractable protein g^{-1} fresh weight for stalk tissue compared with the other tissues.

333 **The high dose refuge *B. thuringiensis* resistance management strategy**

334 One of the challenges for the long term commercial use of transgenic *cry* corn plants
335 is the possibility that ECB could develop resistance against such plants. After 30 years
336 of use of *B. thuringiensis* sprays, only isolated field resistance was found in one insect
337 pest. Resistance development has in fact been more of a problem for synthetic
338 chemical insecticides. Based on theoretical computer modelling studies, the high dose
339 refuge strategy is perceived by many experts as one of the most promising strategies.
340 The principle of high dose refuge strategy is that transgenic plants express high
341 enough levels of insecticidal protein to kill all susceptible and most of the resistant
342 heterozygote insects. The few surviving resistant homozygote adult insects will mate
343 with susceptible adults coming from refugia fields with non-*B. thuringiensis* transgenic
344 plants. The offspring from such a mating will be resistant heterozygotes, which again
345 can quite effectively be controlled by the transgenic plants (Roush, 1994). The number
346 of susceptible insects needed, and therefore the ratio of non-toxic to toxic plants,
347 required for an effective resistance management strategy, is as yet unknown. Refuge
348 requirements will likely be different for each habitat, crop and pest situation (Alstad
349 and Andow, 1995). Although the size of the refugia area has an impact on the
350 resistance development, the capacity of the transgenic plants to eliminate heterozygous

resistant insects is perhaps even more important (Roush, 1994). In most studies, the insecticidal activity of the transgenic plants has been tested with the youngest, most susceptible larvae. However, a certain level of a crystal protein may be a high dose for the youngest larval instars, but may be a low dose for the older larval instars or for heterozygous resistant insects (Gould, 1994). Since ECB populations resistant to *B. thuringiensis* corn events were not available, the insecticidal activity of different Cry9C events was tested with larger instar larvae.

Second, third and fourth instar larvae fed with leaf material of event CBH351 all died within six days (Table 7). For CBH305, which expresses approximately one-tenth less Cry9C protein, only one fourth instar larva survived after eight days. From these data it appears that the CBH351 event provides at least 10 fold the level of Cry9C that would kill 100% of fourth instar ECB larvae. A high dose refuge strategy requires the high dose to exist throughout the growing season, preventing larvae surviving and overwintering in mature corn plants because of decreasing crystal protein levels. At harvest, the concentrations of Cry9C protein for CBH351 were 225, 94 and 18 $\mu g\ g^{-1}$ fresh weight for leaves, tassel and pith, respectively. The LC_{50} of Cry9C protein for neonate ECB larvae is 96.6 ng cm^{-2} diet (Lambert et al. 1996).

Clearly, the Cry9C events with high ECB season long resistance, such as CBH351, can be considered as 'high dose' plants and fit well with a high dose refuge resistance management strategy. In fact, when 30% plants in the plots were non-transgenic, which could be considered as a 30% in-field refugia, CBH351 still gave excellent control.

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462 FIGURE CAPTIONS

463 Figure 1. Stalks of non-transgenic control plants with ECB tunneling (left) and stalks

464 of Cry9C-transgenic plants of event CBH351 (right) from Belgium field trial.

Table 1. Mean (+/-S.D.) ECB1 leaf feeding damage rating using 0-9 modified Guthrie scale, Belgium.

Entry	Genetic Background†	Non-transgenic plants in Cry9C plots				
		not removed			removed	
		Percentage non-transgenic plants	Leaf Damage Rating		Leaf Damage Rating	
Control1H	B73x(H99xCo)	-	5.09(0.83)	k§	4.80(0.75)	k
Control2B	B73x(B73xCo)	-	5.43(0.41)	l	5.72(1.00)	l
ControlH99	H99	-	-		-	
CBH202	B73x(ToxB73)	52.0	1.92(0.63)	f‡	0.91(0.84)	ab
CBH353	H99x(ToxH99)	25.0	1.79(0.86)	ef	1.05(0.90)	ab
CBH201	H99x(H99xTo)	44.4	1.50(0.78)	cdef	1.17(0.57)	b
CBH203B	B73x(H99xTo)	37.5	1.61(0.16)	def	1.34(0.73)	b
CBH305	B73x(B73xTo)	42.0	1.14(0.38)	bcde	1.07(1.18)	b
CBH352	H99x(H99xTo)	17.8	0.44(0.59)	ab	0.32(0.36)	a
CBH351	H99x(H99xTo)	30.5	0.37(0.38)	a	0.61(0.61)	ab
CBH301	H99x(ToxH99)	50.0	0.81(0.38)	abc	0.33(0.10)	a
CBH306	B73x(B73xTo)	39.7	1.48(0.49)	cdef	1.26(0.73)	b
CBH302	B73x(B73xTo)	55.0	0.96(0.90)	abcd	0.74(0.44)	ab

† To is primary event and Co is control plant with (Pa91xH99)xH99 genetic background.

‡ Means within a column followed by the same letter (a,b,c,d,e,f) are not significantly different ($P < 0.05$) by LSD test.

§ Means within a column followed by a letter (k,l,m) are not significantly different ($P < 0.05$) by LSD test from the means of negative control entries denoted by the same letter.

Table 2. ECB2 stalk feeding damage expressed as mean (+/S.D.) number of stalk tunnels per plant and mean (+/-S.D.) total tunnel length per plant, Belgium.

Entry	Number of stalk tunnels per plant				Total stalk tunnel length per plant in cm			
	Non-transgenic plants in Cry9C plots				Non-transgenic plants in Cry9C plots			
	not removed		removed		not removed		removed	
Control1H	7.47(1.83)	k§	6.73(0.79)	k	40.4(18.5)	k	39.6(11.9)	k
Control2B	8.27(2.72)	l	7.70(1.40)	l	49.2(13.5)	l	42.3(17.2)	l
ControlH99	6.25(0.75)	m	5.27(0.49)	m	36.6(1.72)	m	30.7(4.57)	mn
CBH202	5.50(1.97)	b†x‡ klm	3.07(1.59)	cx	29.3(9.71)	cx klm	16.3(7.45)	cy
CBH353	4.80(1.20)	bx m	2.70(1.40)	cx	20.8(15.0)	bcx	15.2(13.0)	cx
CBH201	3.77(1.55)	bx	3.40(2.57)	cx	15.1(4.47)	bx	14.4(10.0)	cx
CBH203B	4.93(2.57)	bx m	2.83(1.39)	cx	21.8(10.4)	bcx m	13.0(6.40)	cy
CBH305	4.63(3.36)	bx m	0.76(0.51)	by	12.8(7.39)	bx	2.19(1.82)	by
CBH352	1.00(0.88)	ax	0.23(0.39)	abx	1.87(1.73)	ax	0.25(0.34)	ay
CBH351	0.80(0.97)	ax	0.10(0.20)	abx	1.45(1.77)	ax	0.14(0.27)	ay
CBH301	1.60(0.80)	ax	0.10(0.00)	ay	1.95(1.10)	ax	0.24(0.19)	ay
CBH306	4.27(2.41)	bx	0.03(0.07)	ay	14.4(1.46)	bx	0.07(0.16)	ay
CBH302	1.47(1.43)	ax	0.03(0.07)	ay	2.97(2.90)	ax	0.04(0.08)	ay

† Means within a column followed by the same letter (a,b,c) are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation.

‡ Means within a row followed by the same letter (x,y) are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation.

§ Means within a column followed by a letter (k,l,m) are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation from the means of negative control entries denoted by the same letter.

Table 3. Mean ECB1 leaf feeding damage using 0-9 modified Guthrie scale and ECB2 stalk feeding damage expressed as mean (+/S.D.) number of stalk tunnels per plant and mean (+/-S.D.) total tunnel length per plant,

Iowa, USA.

		ECB1	ECB2	
Entry	Genetic Background†	Leaf feeding damage rating	Number of stalk tunnels per plant	Total stalk tunnel length per plant in cm
ControlB73	B73	3	8.57 (2.87) d‡	23.8 (4.31) c
CBH202	B73x(ToxB73)	0	6.50 (1.59) cd	18.9 (5.12) c
CBH203B	B73x(H99xTo)	0	5.60 (1.77) bcd	15.1 (5.80) c
CBH305	B73x(B73xTo)	0	3.57 (2.67) b	6.33 (5.90) b
CBH351	B73x(ToxB73)	0	0.06 (0.07) a	0.09 (0.11) a
CBH353	B73x(ToxH99)	0	4.50 (1.80) bc	14.7 (9.99) c

† To is primary transformant with (Pa91xH99)xH99 genetic background

‡ Means within a column followed by the same letter are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation.

Table 4. Mean (+/-S.E.) Cry9C protein concentrations in leaf tissue of Cry9C events at different plant stages, Belgium.

	µg Cry9C protein mg^{-1} soluble protein				
Event	Mid Whorl	Late Whorl	Tasseling	Silking	Harvest
CBH202	0.76(0.19)n=5 †	0.43(0.09)n=6	0.20(0.06)n=6	0.42(0.20)n=6	0.22(0.18)n=6
CBH203	0.20(0.03)n=5	0.10(0.04)n=7	0.09(0.02)n=8	0.13(0.04)n=7	0.05(0.02)n=8
CBH301	19.0(5.11)n=5	14.4(8.08)n=5	10.1(4.58)n=5	8.94(4.93)n=5	3.05(1.44)n=4
CBH305	0.92(0.27)n=5	1.24(0.11)n=5	0.48(0.12)n=6	0.47(0.10)n=7	0.48(0.08)n=6
CBH306	2.00(0.55)n=5	2.42(0.61)n=6	2.68(1.19)n=6	0.32(0.07)n=5	1.63(0.62)n=6
CBH351	32.4(3.28)n=5	39.0(2.23)n=7	25.1(4.41)n=7	24.3(4.85)n=7	39.0(1.93)n=6
CBH353	3.08(1.63)n=5	0.34(0.10)n=5	0.14(0.04)n=5	0.09(0.03)n=7	0.07(0.03)n=3

† n = number of plants

Table 5. Mean (+/-S.E.) Cry9C protein concentrations in various tissues of Cry9C events CBH203 and CBH351 at different plant stages, Belgium.

	µg Cry9C protein mg^{-1} soluble protein			
Tissue	Late Whorl	Tasseling	Silking	Harvest
		Event CBH203		
Tassel	0.27(0.13)n=4 † 0.38(0.14)n=5		1.38(0.43)n=4	0.52(0.27)n=5
Silks	-	-	0.02(0.02)n=4	0.27(0.15)n=4
Kernels	-	-	0.00 n=5	0.05(0.03)n=4
Stalk	-	-	0.08(0.04)n=5	-
Stalk(pith)	-	-	-	0.02(0.02)n=5
Stalk(cortex)	-	-	-	0.02(0.02)n=5
		Event CBH351		
Tassel	11.7(1.67)n=3	24.0(3.00)n=2	17.3(6.42)n=4	17.4(1.50)n=5
Silks	-	-	16.3(6.25)n=4	6.80(0.97)n=5
Kernels	-	-	14.3(2.67)n=3	1.80(0.49)n=5
Stalk	-	-	47.5(15.9)n=4	-
Stalk(pith)	-	-	-	84.8(14.9)n=5
Stalk(cortex)	-	-	-	74.2(17.4)n=5

† n = number of plants

Table 6. Toxicity of corn-expressed and bacterial Cry9C protein to neonate *O. nubilalis* larvae.

Tissue	Plant extract	Total soluble protein $\mu g\ ml^{-1}$	Cry9C protein $\mu g\ ml^{-1}$	LC50 $ng\ cm^{-2}$	FL95Min-Max/Slope
Pith	CBH351-29a	62	6.85	66.4	44.8-103.9/ 2.2
Pith	CBH351-29b	106	7.63	47.3	32.2-67.1/ 2.8
Pith	CBH351-58	103	11.9	61.2	26.0-103.6/ 1.6
Pith	CBH351-83	82	7.63	50.9	21.7-86.5/ 1.6
Pith	Control	275	-	undiluted extract showed no toxicity	
Pith	Control + Cry9C bact. toxin	275	7.63	59.3	26.8-108.3/ 1.3
-	- Cry9C bact. toxin	-	-	35.5	21.4-53.7/ 2.0
Cortex	CBH351-29a	123	17.3	114.2	58.4-166.9/2.7
Cortex	CBH351-29b	190	6.27	33.8	22.0-49.9/2.1
Cortex	CBH351-58	179	15.21	46.0	- /2.2
Cortex	CBH351-83	120	7.56	38.4	22.7-57.8/2.2
Cortex	Control	182	-	undiluted extract showed no toxicity	
Cortex	Control + Cry9C bact. toxin	182	7.64	35.5	13.3-75.6/1.7
-	- Cry9C bact. toxin	-	-	39.1	21.7-57.8/2.5

Table 7. Mean Mortality (+/-S.E.) of ECB larvae of different instars on leaf material of events CBH305 and CBH351.

		Mortality in %			
Plant line	Instar	Day 2	4	6	8
Control	L1	0	0	0	0
	L2	0	0	0	0
	L3	0	0	6.3(3.6)	6.3(3.6)
	L4	0	0	0	2.1(2.1)
CBH351	L1	49.9(3.6)	95.8(2.1)	100	100
	L2	0	4.2(2.1)	100	100
	L3	0	60.4(7.5)	100	100
	L4	14.6(8.3)	85.4(8.4)	100	100
CBH305	L1	27.2(2.2)	97.9(2.1)	100	100
	L2	0	15.3(5.0)	100	100
	L3	0	81.3(6.3)	100	100
	L4	4.2(4.2)	97.9(2.1)	97.9(2.1)	97.9(2.1)

Table 2. ECB2 stalk feeding damage expressed as mean (+/S.D.) number of stalk tunnels per plant and mean (+/-S.D.) total tunnel

length per plant, Belgium.

Entry	Number of stalk tunnels per plant				Total stalk tunnel length per plant in cm			
	Non-transgenic plants in Cry9C plots				Non-transgenic plants in Cry9C plots			
	not removed		removed		not removed		removed	
Control1H	7.47(1.83)	k§	6.73(0.79)	k	40.4(18.5)	k	39.6(11.9)	k
Control2B	8.27(2.72)	l	7.70(1.40)	l	49.2(13.5)	l	42.3(17.2)	l
ControlH99	6.25(0.75)	m	5.27(0.49)	m	36.6(1.72)	m	30.7(4.57)	mn
CBH202	5.50(1.97)	b†x‡ klm	3.07(1.59)	cx	29.3(9.71)	cx klm	16.3(7.45)	cy
CBH353	4.80(1.20)	bx m	2.70(1.40)	cx	20.8(15.0)	bcx	15.2(13.0)	cx
CBH201	3.77(1.55)	bx	3.40(2.57)	cx	15.1(4.47)	bx	14.4(10.0)	cx
CBH203B	4.93(2.57)	bx m	2.83(1.39)	cx	21.8(10.4)	bcx m	13.0(6.40)	cy
CBH305	4.63(3.36)	bx m	0.76(0.51)	by	12.8(7.39)	bx	2.19(1.82)	by
CBH352	1.00(0.88)	ax	0.23(0.39)	abx	1.87(1.73)	ax	0.25(0.34)	ay
CBH351	0.80(0.97)	ax	0.10(0.20)	abx	1.45(1.77)	ax	0.14(0.27)	ay
CBH301	1.60(0.80)	ax	0.10(0.00)	ay	1.95(1.10)	ax	0.24(0.19)	ay
CBH306	4.27(2.41)	bx	0.03(0.07)	ay	14.4(1.46)	bx	0.07(0.16)	ay
CBH302	1.47(1.43)	ax	0.03(0.07)	ay	2.97(2.90)	ax	0.04(0.08)	ay

† Means within a column followed by the same letter (a,b,c) are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation.

‡ Means within a row followed by the same letter (x,y) are not significantly different ($P < 0.05$) by LSD test after log(x + 1) transformation.

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Table 1. Mean (+/-S.D.) ECB1 leaf feeding damage rating using 0-9 modified Guthrie scale, Belgium.

Entry	Genetic Background†	Non-transgenic plants in Cry9C plots				
		not removed			removed	
		Percentage non-transgenic plants	Leaf Damage Rating		Leaf Damage Rating	
Control1H	B73x(H99xCo)	-	5.09(0.83)	k§	4.80(0.75)	k
Control2B	B73x(B73xCo)	-	5.43(0.41)	l	5.72(1.00)	l
ControlH99	H99	-	-		-	
CBH202	B73x(ToxB73)	52.0	1.92(0.63)	f‡	0.91(0.84)	ab
CBH353	H99x(ToxH99)	25.0	1.79(0.86)	ef	1.05(0.90)	ab
CBH201	H99x(H99xTo)	44.4	1.50(0.78)	cdef	1.17(0.57)	b
CBH203B	B73x(H99xTo)	37.5	1.61(0.16)	def	1.34(0.73)	b
CBH305	B73x(B73xTo)	42.0	1.14(0.38)	bcde	1.07(1.18)	b
CBH352	H99x(H99xTo)	17.8	0.44(0.59)	ab	0.32(0.36)	a
CBH351	H99x(H99xTo)	30.5	0.37(0.38)	a	0.61(0.61)	ab
CBH301	H99x(ToxH99)	50.0	0.81(0.38)	abc	0.33(0.10)	a
CBH306	B73x(B73xTo)	39.7	1.48(0.49)	cdef	1.26(0.73)	b
CBH302	B73x(B73xTo)	55.0	0.96(0.90)	abcd	0.74(0.44)	ab

† To is primary event and Co is control plant with (Pa91xH99)xH99 genetic background.

‡ Means within a column followed by the same letter (a,b,c,d,e,f) are not significantly different ($P < 0.05$) by LSD test.

§ Means within a column followed by a letter (k,l,m) are not significantly different ($P < 0.05$) by LSD test from the means of negative control entries denoted by the same letter.

AgrEvo

Title

Composition of Grain from Cry9C Corn Derived from Transformation Event CBH-351, USA 1996

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Summary:

Aspects of grain composition including crude fat, protein, fiber, and ash content; amino acid profile; and Ca and P content of Cry9C corn hybrids were compared to non-transgenic counterparts and to a USDA database. All aspects of grain composition measured were within CODEX standards. With the exception of crude protein and crude fibre, hybrids expressing the Cry9C protein were not different than their non-transgenic counterpart.

Introduction:

Cry9C corn expresses an insecticidal protein derived from *Bacillus thuringiensis* subspecies *tolworthi*. The Cry9C protein bestows protection from the European Corn Borer, an economically significant pest of corn in the USA.

To demonstrate that neither the transgene products nor the transformation process has negatively impacted the nutritional or compositional value of corn grain harvested from Cry9C corn, 1996 field grown grain samples harvested from five Cry9C hybrids were analyzed for various compositional properties and were compared to non-transgenic counterparts and related standard hybrids. The results of these analyses are presented below.

Methods:

Field phase: Five different hybrids of Cry9C corn (designated +Bt), their non-transgenic counterparts (designated -Bt), and standard hybrids (designated Std) were field grown in Iowa in 1996. The relationship between the +Bt hybrid, the -Bt hybrid and the Std hybrid in each of the five sets can be described as follows:

+Bt hybrid:	Line A x Line B) (Line C x Line +Bt
- Bt hybrid:	Line A x Line B) (Line C x Line -Bt
Std hybrid:	Line A x Line B) (Line C, or
	Line A x Line B, or
	Line A x Line C.

Therefore, the +Bt and the -Bt hybrids share 3 of 4 parental lines while the Std hybird shares 2 or 3 of 4 parental lines with the others.

At maturity, grain was harvested from each hybrid and sent to Woodsen-Tenent Laboratories, Des Moines, IA for compositional analysis.

Analytical phase: The following standard methods were used to determine the composition of the grain:

(1) moisture: AOCS Ba 2a-38
(2) ash: AOAC 15th Ed. 1990, 942.05
(3) fat, crude: AOAC 15th Ed. 1990, 920.39
(4) fibre, crude: AOAC 15th Ed. 1990, 962.09.
(5) protein, Kjeldahl: AOCS Ba 4d-90
(6) calcium: AOAC 15th Ed. 1990, 968.08 Modified
(7) phosphorus: AOAC 15th Ed. 1990, 965.17
(8) amino acid profile: AOAC 15th Ed. 1990, 982.30 D, E, and F.

Statistical analyses: Proximate analyses data of corn hybrids expressing the Cry9C protein (+Bt) were compared to their non-transgenic counterparts (-Bt) using a paired t-test. All raw data and statistical analyses are presented in Appendix 1.

Results and Discussion:

Proximate analyses data showed that corn grain from Cry9C corn hybrids (+Bt) differed from their non-transgenic conterparts in protein and fibre content only (Table 1). With the exception of protein and ash content which were higher, all values obtained from the proximate analysis fell within a 95% confidence interval calculated from USDA data (Human Nutrition Information Service, 1989). In each case, the non-transgenic counterpart (-Bt) and the related standard hybrid (Std) were also above the confidence interval. This indicates that neither the transgene product nor the transformation process account for the value outside of the reference range.

Table 1. Results of proximate analysis conducted on 5 hybrids. Values represent the mean of determinations on 5 different hybrids in each category. Dry weight basis data was used for statistical comparison of means.

%Content	Wet or dry wt. basis	+Bt[a]	-Bt	Std	USDA - HNIS Reference[b]
Moisture	wet	9.51	9.42	9.46	8.03 - 12.71
	dry	n.a.	n.a.	n.a.	-
Fat / Oil	wet	3.81	3.70	3.61	3.29 - 6.19
	dry	4.21	4.09	3.98	-
Protein	wet	10.38	10.97	10.77	8.6 - 10.24
	dry	11.47*	12.11	11.89	-
Fibre	wet	1.86	1.76	1.92	0.36 - 5.44
	dry	2.06*	1.94	2.12	-
Ash	wet	1.7	1.65	1.76	0.98 - 1.44
	dry	1.87	1.82	1.94	-

[a]Values in the +Bt column followed by an asterisk indicate a significant difference in the means (P = 0.05) between the +Bt and the -Bt hybrids according to a paired two-sample t-test.

[b]Range presented is 95% confidence interval calculated from UDSA-HNIS data (1989).
For many amino acids, the % content determined in the grain of Cry9C corn (+Bt), the non-transgenic counterpart (-Bt), and the related standard hybrid (Std) are higher than the reference value (Table 2). Taking into consideration the relatively higher protein levels found in these hybrids (Table 1), this result is not surprising. The reference value is a mean taken from a USDA database (Human Nutrition Information Service, 1989). The mean is representative of 16 to 101 samples depending upon the particular amino acid, however no indication of variability was included with the data. The differences in the amino acid profile among the corn hybrids tested and the reference value are not large and likely do not represent a significant departure from what would be considered normal for corn grain. Furthermore, the differences shown in amino acid profiles do not appear to be related to whether or not the hybrid expresses the Cry9C protein.

Table 2. Amino acid profile of a Cry9C corn hybrid (+Bt), its non-transgenic counterpart (-Bt) and a related standard hybrid (Std).

Amino acid profile	Reference (USDA - HNIS Data)	% Content in Seed		
		+Bt	-Bt	Std
Tryptophan	0.057	0.06	0.06	0.06
Aspartic acid	0.565	0.61	0.71	0.63
Threonine	0.305	0.33	0.39	0.33
Serine	0.386	0.47	0.57	0.48
Glutamic acid	1.525	1.74	2.15	1.78
Proline	0.709	0.84	1.00	0.85
Glycine	0.333	0.32	0.36	0.32
Alanine	0.608	0.71	0.86	0.73
Cystine	0.146	0.20	0.20	0.18
Valine	0.411	0.41	0.46	0.41
Methionine	0.170	0.19	0.21	0.18
Isoleucine	0.291	0.31	0.37	0.32
Leucine	0.996	1.22	1.52	1.25
Tyrosine	0.330	0.18	0.22	0.18
Phenylalanine	0.399	0.46	0.56	0.47
Histidine	0.248	0.30	0.34	0.29
Lysine, total	0.228	0.24	0.26	0.24
Arginine	0.405	0.36	0.40	0.36

The calcium and phosphorus content in grain from all three hybrids (Table 3) fell within the 95% confidence interval calculated from USDA data (Human Nutrition Information Service, 1989): 0.004 to 0.010% for calcium and 0.13 to 0.29% for phosphorus.

Table 3. Calcium and phosphorus content of a Cry9C corn hybrid (+Bt), its non-transgenic counterpart (-Bt) and a related standard hybrid (Std).

Hybird	% Calcium	% Phosphorus
+Bt	0.009	0.28
-Bt	0.009	0.28
Std	0.009	0.27

Conclusion:

Based on the data presented above, there is no indication that either the transgene products expressed in grain from Cry9C corn hybrids or the transformation process itself has had a negative impact on the nutritional quality of the grain.

Reference:

Human Nutrition Information Service. 1989. Composition of Foods: Cereal Grains and Pasta, Raw, Processed, Prepared. Agriculture Handbook Number 8-20. Washington, DC: U.S. Department of Agriculture.

APPENDIX:

RAW DATA AND STATISTICS TABLES FOR PROXIMATE ANALYSES

Raw data		% content in seed					adjusted to dry wt. basis			
		moisture	fat/oil	protein	fiber	ash	fat / oil	protein	fiber	ash
Hybrid #1	Std	9.36	3.97	10.17	1.7	1.43	4.38	11.22	1.88	1.58
	+Bt	9.68	4.05	10.97	1.8	1.37	4.48	12.15	1.99	1.52
	-Bt	9.42	4.01	11.22	1.6	1.45	4.43	12.39	1.77	1.60
Hybrid #2	Std	9.55	4.14	10.05	1.9	1.74	4.58	11.11	2.10	1.92
	+Bt	9.36	3.95	10.37	1.8	1.61	4.36	11.44	1.99	1.78
	-Bt	9.29	3.85	11.45	1.7	1.74	4.24	12.62	1.87	1.92
Hybrid #3	Std	9.46	3.32	11	2.2	1.63	3.67	12.15	2.43	1.80
	+Bt	9.6	3.57	10.03	1.9	1.74	3.95	11.10	2.10	1.92
	-Bt	9.49	3.67	10.61	1.9	1.63	4.05	11.72	2.10	1.80
Hybrid #4	Std	9.56	2.96	11.27	2	2.04	3.27	12.46	2.21	2.26
	+Bt	9.59	3.44	10.46	1.9	1.97	3.80	11.57	2.10	2.18
	-Bt	9.43	3.35	10.8	1.9	1.52	3.70	11.92	2.10	1.68
Hybrid #5	Std	9.38	3.65	11.34	1.8	1.95	4.03	12.51	1.99	2.15
	+Bt	9.31	4.06	10.05	1.9	1.79	4.48	11.08	2.10	1.97
	-Bt	9.48	3.64	10.76	1.7	1.91	4.02	11.89	1.88	2.11
Means	Std.	9.46	3.61	10.77	1.92	1.76	3.98	11.89	2.12	1.94
	+Bt	9.51	3.81	10.38	1.86	1.70	4.21	11.47	2.06	1.87
	-Bt	9.42	3.70	10.97	1.76	1.65	4.09	12.11	1.94	1.82

Fat content

	+Bt	-Bt
Hybrid #1	4.48	4.43
Hybrid #2	4.36	4.24
Hybrid #3	3.95	4.05
Hybrid #4	3.8	3.7
Hybrid #5	4.48	4.02

t-Test: Paired Two Sample for Means

	+Bt	*-Bt*
Mean	4.214	4.088
Variance	0.10098	0.07417
Observations	5	5
Pearson Correlation	0.767653	
Hypothesized Mean Difference	0	
df	4	
t Stat	1.370212	
P(T<=t) one-tail	0.121244	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.242489	
t Critical two-tail	2.776450	

Protein content

	+Bt	-Bt
Hybrid #1	12.15	12.39
Hybrid #2	11.44	12.62
Hybrid #3	11.1	11.72
Hybrid #4	11.57	11.92
Hybrid #5	11.08	11.89

t-Test: Paired Two Sample for Means

	+Bt	*-Bt*
Mean	11.468	12.108
Variance	0.19057	0.14377
Observations	5	5
Pearson Correlation	0.583268	
Hypothesized Mean Difference	0	
df	4	
t Stat	-3.807770	
P(T<=t) one-tail	0.009488	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.018976	
t Critical two-tail	2.776450	

Fibre content

	+Bt	-Bt
Hybrid #1	1.99	1.77
Hybrid #2	1.99	1.87
Hybrid #3	2.1	2.1
Hybrid #4	2.1	2.1
Hybrid #5	2.1	1.88

t-Test: Paired Two Sample for Means

	+Bt	*-Bt*
Mean	2.056	1.944
Variance	0.00363	0.02213
Observations	5	5
Pearson Correlation	0.760922	
Hypothesized Mean Difference	0	
df	4	
t Stat	2.274844	
P(T<=t) one-tail	0.042635	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.085270	
t Critical two-tail	2.776450	

Ash content

	+Bt	-Bt
Hybrid #1	1.52	1.6
Hybrid #2	1.78	1.92
Hybrid #3	1.92	1.8
Hybrid #4	2.18	1.68
Hybrid #5	1.97	2.11

t-Test: Paired Two Sample for Means

	+Bt	*-Bt*
Mean	1.874	1.822
Variance	0.05978	0.04062
Observations	5	5
Pearson Correlation	0.266653	
Hypothesized Mean Difference	0	
df	4	
t Stat	0.427091	
P(T<=t) one-tail	0.345649	
t Critical one-tail	2.131846	
P(T<=t) two-tail	0.691298	
t Critical two-tail	2.776450	

FINAL REPORT

Study Title

MOLECULAR CHARACTERIZATION OF THE CRY9C CORN TRANSFORMATION EVENT CBH-351

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MOLECULAR CHARACTERIZATION OF THE CRY9C CORN TRANSFORMATION EVENT CBH-351

I. Purpose

Plant Genetic Systems has developed corn (*Zea mays*) plants that express an insecticidal protein Cry9C from a soil bacterium, *Bacillus thuringiensis* subsp. *tolworthi (Bt tolworthi)*. The Cry9C is effective in controlling lepidopteran larvae such as European corn borer (ECB) (*Ostrinia nubilalis (Huber))* larvae, which is a common pest of corn. ECB damage currently can cost farmers 5 to 20% loss. When chemical insecticidal sprays are used, the protection is only partial, due to the biology of the pest species. Field trials in the US and Europe with transgenic corn plants expressing the Cry9C protein, have shown excellent control of ECB larvae. Cry9C is a newly discovered protein which has not previously been used commercially. Cry9C action in the insect midgut is mediated through interaction with a novel binding site.

These plants also contain an insecticidal inert trait as selectable marker, the phosphinothricin acetyltransferase (PAT) protein, which confers tolerance to glufosinate-ammonium containing herbicides.

The purpose of this section is to characterize the inserted DNA in the corn transformation event CBH-351. Based on Southern analysis the inserted gene copy number has been determined. Northern analysis provides information on the expression of the transgenes and includes the evaluation of potential cryptic gene expression. Molecular and genetic data in the stability of transformation event CBH-351 are presented.

II. Material and Methods

Southern analysis

The Southern blotting procedure permits a rapid and precise analysis of the size distribution of specific DNA sequences among a mixture of many different sequences. Southern hybridization has become a routine procedure to detect chimeric genes and to determine the number of the genes that are inserted into the transgenic plant genome (Southern, 1975).

Southern blot analysis with plant material is generally performed according to the protocol described by Sambrook *et al.* (1989) and Dellaporta *et al.* (1983). Genomic DNA is isolated and digested with appropriate restriction endonucleases, which are enzymes that selectively cleave DNA at specific nucleotide sequences. Using gel electrophoresis, the resulting DNA fragments are separated by size through a gel matrix. After the electrophoresis, the DNA is denatured and transferred to a solid membrane (nylon filters). When complementary nucleic acid sequences of the target gene are added as probes to the membrane, the DNA fragments are able to form double-stranded molecules with immobilized counterparts through DNA:DNA hybridization. The radioactively labeled DNA fragments serve as a means for detecting and identifying specific gene

sequences. The respective DNA sequences can be visualized by autoradiography (Southern, 1975; Sambrook *et al.*, 1989).

Preparation of total genomic DNA

- Collect between 0.5 and 1g of leaf tissue, freeze in liquid nitrogen, grind to a fine powder in a mortar with a pestle, and transfer the powder into a 30ml Oak Ridge tube containing 15ml extraction buffer (100mM Tris.HCl pH 8, 50mM EDTA, 500mM NaCl, 10mM ß mercaptoethanol).
- Add 1ml of 20% SDS, mix thoroughly by vigorous shaking and incubate the tubes at 65°C for 10 min.
- Add 5ml of 5M potassium acetate, shake tubes vigorously and incubate at 0°C for about 20 min.
- Spin tubes at 25000xg for 20 min (13000 rpm in Sorvall SA 600 rotor). Pour supernatant through a Miracloth filter (Calbiochem) into a clean 30ml tube, containing 10ml isopropanol. Mix and incubate at -20°C for 30 min .
- Pellet the DNA at 20000xg for 15 min. Gently pour off the supernatant and dry pellets by inverting the tubes on paper towels for 10 min.
- Redissolve DNA pellets with 700 µl of TE20 buffer (50mM Tris.HCl pH 8, 20mM EDTA), and transfer to a microfuge tube.
- Add 5 µl RNase (10 mg/ml) and incubate for 10 min at 37°C.
- Spin tubes for 10 min in a microfuge to remove insoluble debris.
- Transfer the supernatant to a new eppendorf tube and add 75 µl 3M sodium acetate and 500µl isopropanol. Mix well and pellet the DNA for 30 seconds in a microfuge.
- Wash pellets with 80% ethanol, dry and redissolve DNA in 100 µl TE buffer (10mM Tris.HCl pH 8, 1mM EDTA).
- Determine the concentration of the DNA by measuring the UV absorbance at 260 nm. An OD of 1 corresponds to 50µg/ml DNA.

Restriction digests of total genomic DNA

- 5 or 10µg genomic DNA are digested in a total volume of 50µl.
- Mix in a microfuge tube : 10µg of genomic DNA
  5µl 10xRE buffer (*)
  10 to 20 units of restriction enzyme
  H_2O up to 50 µl
- Incubate the digest overnight at the recommended temperature.
- Add 5µl of gel-loading buffer.

(*): composition of 10 x RE buffer
- 100mM Tris.HCl pH 8
- 50mM $MgCl_2$
- 60mM ß mercaptoethanol
- 1mM EDTA
- 1mg/ml BSA

Supplemented with 0.5M NaCl (RE50), 1M NaCl (RE100) or 1.5M NaCl (RE150). Or alternatively, the buffer recommended by the manufacturer was used.

Separation of the restriction fragments on agarose gels

- Prepare 1% agarose gel in TAE buffer (40mM Tris, 5mM sodium acetate, 1mM EDTA, pH 7.8 with acetic acid), containing 0.3 µg/ml Ethidium Bromide.
- Pour the gel into a, preferably, horizontal gel support and let solidify.
- Load the DNA samples into the wells of the gel. Include a MW marker (l-DNA digested with PstI, or commercial available MW ladder, such as the 1Kb ladder from BRL-Life technologies).
- Run the gel slowly (1V/cm) overnight .
- Cover the gel with Saran-wrap after the samples have migrated about 1 cm into the gel.

Blotting of the restriction fragments to nylon membranes

- After electrophoresis is completed, cut the gel from the support. Place a fluorescent ruler alongside the gel and document the fractionation of the DNA. The image is acquired, processed and copied to thermal paper using the Foto/Analyst™ Visionary imaging system from FOTODYNE (CCD camera: charge-coupled device).
- Blot the separated DNA fragments to Hybond-N+(Amersham). Hybond-N+is a positively charged nylon membrane which yields excellent sensitivity in both alkali blotting and conventional Southern blotting.

Southern blotting

- Depurinate the gel in 0.25M HCl until the bromophenol blue changes color.
- Rinse the gel with water. Place the gel in denaturation solution (1.5M NaCl, 0.5M NaOH) for 30 to 45 min.
- Rinse the gel with water. Place the gel in neutralization solution (1.5M NaCl, 0.5M Tris-HCl, pH7.2, 0.001M EDTA) for 30 to 45 min.
- Rinse the gel with water and set up the capillary blot using 20xSSC (3M NaCl, 0.3M Sodium citrate) as blotting buffer.

Alkali blotting

- Depurinate the gel in 0.25M HCl until the bromophenol blue changes color.
- Rinse the gel with water and set up the capillary blot using 0.4M NaOH as blotting buffer.

Capillary blotting

- Fill a glass dish with blotting buffer (Either 20xSSC or 0.4M NaOH). Make a platform and cover it with a Whatman 3MM filter paper wick, saturated with buffer.

- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with SaranWrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.
- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1Kg weight on top. Allow the transfer to proceed for 8 to 16 hours.
- After blotting carefully, dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- Rinse the membrane in 2xSSC. Air dry the membrane.
- For capillary blotting using 20xSSC: fix the DNA to the membrane by baking in an oven at 80°C for 2 hours. For alkali blots: there is no need to fix DNA after alkali blotting.

Purification of fragments for probe preparation

- Digest ±20µg of the plasmid DNA with the appropriate restriction enzyme.
- Separate the DNA fragments on a 1% Low Melting Agarose gel, prepared in TAE buffer, containing 0.3 µg/ml Ethidium bromide.
- After electrophoresis is completed, cut the desired fragment from the gel with a scalpel. Put the gel slice in an Eppendorf tube.
- Add an equal volume of TE buffer (10mM Tris.HCl pH 8 , 1mM EDTA).
- Melt the gel slice in a 65°C waterbath for 10 min.
- Preheat an equal volume of phenol (equilibrated with TE buffer) 30 sec. at 65°C.
- Add the phenol to the melted gel slice and shake the mixture for 15 min.
- Centrifuge for 10 min in a microfuge to separate the two phases.
- Transfer the water phase to a new Eppendorf tube and extract for a second time with an equal volume of phenol.
- Precipitate the DNA from the water phase with 0.1 volume of 5M Sodium perchlorate and 1 volume of isopropanol.
- Pellet the precipitated DNA by spinning for 15 min in a microfuge.
- Dry pellets and redissolve in 50µl of TE.
- Measure the concentration of the DNA solution.

DNA Labelling

Feinberg and Vogelstein (1984) introduced the use of random sequence hexanucleotides to prime DNA synthesis on denaturated template DNA at numerous sites along its length. Amersham International (Buckinghamshire, UK) has developed the Rediprime DNA labelling system, using nonamer primers, for extra convenience and performance. The system provides individually dispensed reaction mixes which are dried in the presence of a stabilizer and a dye. This makes labelling probes easier and more reproducible.

- Dilute the DNA to be labelled to a concentration of 2.5 - 25ng in 45µl of sterile water.
- Denature the DNA sample by heating to 95 - 100°C for 5 minutes in a boiling water bath.
- Centrifuge briefly and add the denaturated DNA to the labelling mix and reconstitute the mix by gently flicking the tube until the blue color is evenly distributed.
- Centrifuge briefly.
- Add 5µl of Redivue [^{32}P]dCTP and mix by gently pipetting up and down.
- Centrifuge briefly and incubate the tube at 37°C for 10 minutes.
- Removal of unincorporated nucleotides is sometimes desirable to reduce background during hybridization. Probes can be purified by Sephadex™ chromatography or selective precipitation.

Hybridization and autoradiography

- The hybridization and washing steps are carried out in an hybridization oven with rotating bottle rack.
- Prehybridize the filters for 1 - 2 hours in 6xSSC, 5x Denhardt's, 0.5% SDS and 100µg/ml carrier DNA at 65°C.

  20xSSC: 3M NaCl, 0.3M Sodium citrate
  100xDenhardt's solution: 2%(w/v) BSA, 2%(w/v) ficoll and 2%(w/v) Polyvinylpyrrolidone

- Denature the labelled probe by heating for 5 min. at 95°C.
- Remove the hybridization solution from the bottle. Add new hybridization solution together with the denatured radiolabeled probe to the tube and continue the incubation over night (use 5 to 10ml per 200cm^2 of membrane).
- Wash the filters for 5 min. in 6xSSC, followed by 2 washes of 20 to 40 minutes each in 2xSSC, 0.1%SDS. A high stringency wash can be done when the background signal is still unacceptably high: wash the membrane between 5 and 10 minutes in 0.1xSSC, 0.1%SDS solution.
- Remove excess washing solution from the membrane and wrap in Saran-wrap.
- Establish an autoradiograph by exposing the filter for an appropriate time period (usually between 12 and 24 hours) to X-Ray film (Kodak-Xomat) at -70°C with an intensifying screen.

Northern analysis

With Northern blotting, RNA products of specific genes can be characterized with regard to size and relative quantity. The Northern blot technique is similar to Southern blot analysis, with the exception that instead of digested DNA, intact RNA is separated in a gel matrix and transferred to a solid membrane. The transcripts of interest are detected by hybridization to a nucleic acid probe with homologous sequences. Northern blot analysis has become a routine procedure to determine the expression profile of specific genes (Sambrook *et al.*, 1989).

Extraction and purification of total RNA

Total RNAs are isolated according to Jones *et al.* (1985).

- Grind 1 to 2 grams of tissue to a fine powder in liquid nitrogen.
- Add 9 ml of NTES buffer (0.1M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA, 1% SDS) and 6 ml of phenol/chloroform/isoamylalcohol (24:24:1).
- Vortex intensively (approximately 10 min.) in 50 ml Falcon tubes.
- Transfer to a DEPC-treated 30 ml Corex tube and centrifuge in the HB4 Sorvall rotor at 8000 rpm for 10 min.
- Take the aqueous phase, add 1/10 volume of 2M NaOAc and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate at 8000 rpm for 10 min. (HB4, Corex tubes).
- Rinse the pellet with 70% ethanol.
- Dissolve the pellet in 2 ml water. Spin 5 min. at 5000 rpm (HB4 rotor) to sediment impurities.
- Transfer supernatant to a 15 ml Corex tube and add 2 ml 4M Lithium Acetate or 4M Lithium Chloride.
- Leave on ice for at least 3 hours (preferable over night).
- Pellet the precipitate as above and dissolve the pellet in 1.8ml water. Add 0.2ml 2M NaOAc pH 4.8 and add 2 volumes ethanol.
- Mix well and keep at least 1 hour at -20°C.
- Pellet the precipitate as above and rinse pellet with 70% ethanol and invert the tubes to dry the pellet.
- Finally dissolve the pellet in 100 to 500µl water.

This method is scaled down for the extraction of RNA from dry seeds and germinating seeds. For quantitating the amount of RNA, spectrophotometric readings are taken at a wavelength of 260 nm. An OD of 1 corresponds to 40µg/ml RNA.

In vitro synthesis of control RNA transcripts

Control mRNA samples are produced by *in vitro* transcription of linearized plasmid DNA containing the Cry9C gene flanked on one side by the SP6 promoter, on the other side by the T7 promoter. Incubation with the corresponding SP6 or T7 polymerase yield mRNA that can be used as concentration markers in a dilution series. For conditions, see below in "*In vitro* labelling of RNA probes".

Fractionation of RNA

The RNA is separated according to size by electrophoresis through a denaturing agarose gel containing formaldehyde. The gels are prepared by melting agarose (1.5% final concentration) in water, cooling it to 60°C, adding 10x formaldehyde gel-running buffer (0.2M MOPS, 0.05M NaOAc pH7.0 and 0.01M EDTA) and formaldehyde to give a final concentration of 1x and 2.2M respectively. Cast the gels in a chemical hood and allow the gel to set at least for 30 min. at room temperature.
Samples are prepared by mixing the following in a sterile microfuge tube:

- RNA (5µg or 10µg) x µl
- 10x formaldehyde gel-running buffer 2 µl

- formaldehyde 3.5 µl
- formamide 10 µl
- Ethidium bromide (1mg/ml) 1 µl
- H_2O up to 20µl

Note: The control RNA dilutions are complemented with 5µg or 10µg control leaf RNA

Incubate the samples for 15 minutes at 55°C and then chill them on ice. Add 2 µl of sterile DEPC-treated dye (50% glycerol, 0.5% bromophenol blue and 0.5% xylene cyanol FF). Run the gel submerged in 1x formaldehyde gel-running buffer at ±5 V/cm.

Transfer of denatured RNA to nylon membranes

The RNAs are transferred immediately after electrophoresis from the agarose to nylon membranes (Hybond-N, Amersham) by capillary elution.

- Fill a glass dish with blotting buffer (20x SSPE = 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH 7.7). Make a platform and cover with a Whatman 3MM filter paper wick, saturated with buffer.
- Place the gel on the wick and avoid trapping air bubbles beneath it. A sheet of Hybond-N membrane, cut to the exact size of the gel, is placed on top of the gel. Avoid trapping bubbles beneath the membrane.
- Place a sheet of Whatman 3MM cut to size and wetted with blotting buffer, on top of the Hybond-N membrane.
- Surround the gel with Saran Wrap foil to prevent the blotting buffer being absorbed directly into the paper towels above.
- Place a stack of absorbent paper towels on top of the 3MM paper.
- Place a glass plate on top of the paper towels and a 0.5 - 1 Kg weight on top. Allow the transfer to proceed for 12 to 20 hours.
- After blotting carefully dismantle the setup. Before removing from the gel, mark the membrane with a pencil to allow later identification of the tracks.
- The samples are fixed to the membrane by baking in an oven at 80°C for 2 hours.

Documentation of the fractionation of the RNA is done at this stage. The image is acquired, processed and copied to thermal paper using the Foto/Analyst ™ Visionary imaging system from FOTODYNE.

In vitro synthesis of RNA probes

Single-stranded RNA probes of high specific activity are prepared by using as template DNA either plasmid vectors containing polycloning sites downstream from powerful promoters derived from the *Salmonella typhimurium* bacteriophage SP6 or from the *Escherichia coli* bacteriophage T7 or by either using PCR generated templates with 5' extensions containing the sequences from the before mentioned promoters.

In vitro labeling

- Mix the following components in the order given in a microfuge tube at room temperature:

DEPC-treated water	up to 20 µl total volume
Template DNA	500 ng
10x Transcription buffer	2 µl
NTP mix (-UTP), 2.5mM each	3 µl
1mM UTP	1 µl
0.2M DTT	1 µl
RNAse inhibitor (25 units/µl)	1 µl
[a-^{32}P]UTP (20mCi/ml)	5 µl
Bacteriophage DNA-dependent RNA polymerase (7-12 units/µl)	1 µl

(10x Transcription Buffer: 400mM Tris-HCl pH7.5 at 37°C, 60mM $MgCl_2$, 20mM spermidine and 50mM NaCl).

- Mix the reagents by gentle tapping.
- Incubate the reaction for 1 hour at 40°C (SP6 RNA polymerase) or 37°C (T7 RNA polymerase).
- Add 1 µl RNAse inhibitor and 1 µl of RNAse-free pancreatic DNAseI (20 units/µl). Mix and incubate for 15 min. at 37°C.
- Analyze 0.5 µl on a 6% denaturing acrylamide gel.
- The rapid removal of unincorporated nucleotides from the labeling reaction is done by using Bio-spin® 30 chromatography columns (Bio-Gel P-30 polyacrylamide gel, Bio-Rad).

Hybridization and autoradiography

- The filters are prehybridized for 1-2 hours in a hybridization oven using 10ml prehybridization buffer (for 3 filters of 14cm x 19cm) at 65°C.
  Prehybridization buffer: 50% formamide, 5x SSPE, 5x Denhardt's, 0.1% SDS and 100µg/ml carrier DNA at 65°C.

  (20x SSPE: 3.6M NaCl, 0.2M Sodium phosphate, 0.02M EDTA pH7.7)
  (100x Denhardt's solution: 2% (w/v) BSA, 2% (w/v) ficoll and 2% (w/v) Polyvinylpyrrolidone)

- Remove the prehybridization buffer.
- Add fresh prehybridization buffer supplemented with the denaturated radiolabeled probe to the hybridization tube and continue the incubation overnight.
- Wash the filters for 5 min. in 5x SSPE, followed by 2-3 washes of 20-30 minutes each in 2x SSPE, 0.1% SDS and 1 wash of 10-20 minutes in 0.1x SSPE, 0.1% SDS.
- Establish an autoradiography by exposing the filter for 3 up to 96 hours to X-ray film at -70°C with an intensifying screen. The shorter exposures are performed for accurate quantification and for reproduction of the results. The longer exposures are performed to

assure the absence of any signals in control samples or in the analysis of occurrence of cryptic gene expression.
- Reproduction of the results in this document is done by using the photo deluxe software (U-lead Systems, Taipei, Taiwan, ROC) and the Harvard Graphics Software.
- After the exposure, the membranes are stripped to remove the probes. For this purpose a 0.5% SDS solution is boiled. Membranes are submerged in this solution and allowed to cool to room temperature.
- To check that the probe was removed completely, an autoradiograph for the normal exposure time was established.
- Subsequently, the filters can be prehybridized and hybridized with a new probe.

Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) allows sequence specific and highly sensitive detection and amplification of individual DNA sequences from very small initial sample quantities (White *et al.*, 1989).

The polymerase chain reaction is based on the enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. Since the primers are oriented with their 3' end towards each other, repeated cycles, annealing of the primers to their complementary sequences and extension of the annealed primers with a DNA polymerase, will result in the amplification of the segment defined by the 5' ends of the PCR primers. Since the extension product of each primer can serve as a template for the other primer, each cycle essentially doubles the amount of the DNA fragment of interest produced in the previous cycle. This results in the exponential accumulation of the specific target fragment to several million fold in a few hours. Using specific primers, the fragments of specific genes can be amplified. The isolation of the thermostable Taq DNA polymerase of the thermophilic bacterium *Thermus aquaticus*, has led to the possibility to automate the procedure (White *et al.*, 1989). The amplified product can be visualized by agarose gel electrophoresis and/or Southern blotting.

The PCR method can be used to test transgenic plants for the presence of our target genes (*bar and cry9C*). It is a sensitive method and only low amounts of genomic DNA (200ng) are required.

Preparation of Plant Genomic DNA

The rapid extraction of small amounts of plant genomic DNA suitable for PCR analysis is done according to the method described by Edwards *et al.*(1991).
- Collect samples for PCR analysis (usually leaf tissue) by using the lid of a Eppendorf tube to pinch out a disc of material into the tube.
- Macerate the tissue with a plastic pestle at room temperature, without buffer for 5 to 15 sec.
- Add 400 µl extraction buffer. (EB: 200 mM Tris HCl pH 7.5, 250 mM NaCl, 25mM EDTA, 0.5% SDS). The mixture can be left at room temperature until all samples have been extracted (> 1 hour).
- Centrifuge the extracts for 1 minute at max. speed and transfer 300 µl of the supernatant to a fresh Eppendorf tube.

- Mix with 300 µl isopropanol and leave at room temperature for 2 minutes.
- Centrifuge at max. speed for 5 minutes.
- Dry pellet and dissolve in 100 µl water.
- Centrifuge for 2 minutes and transfer supernatant to a new Eppendorf tube.
- Use 5 µl of this sample in a 50 µl PCR reaction.

Polymerase chain reaction

Standard procedure

5µl of the isolated DNA is used in a 50µl PCR reaction containing 10 mM Tris-HCl (pH8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 200µM of each dNTP; 0.001% (w/v) gelatin; 1 unit *Taq* DNA polymerase (Boehringer Mannheim); 10 pmole each of the downstream and upstream oligonucleotide primers. A master mix of reagents (water, buffer, dNTP's, primers and enzyme) for all samples is prepared first and then aliquoted to the individual samples. The reaction mixtures are overlayed with 50µl mineral oil and thermocycling is started.

Thermocycling profile:	4 min. at 95°C	
	Followed by:	1 min. at 95°C
		1 min. at 57°C
		2 min. at 72°C
		For 5 cycles
	Followed by:	30 sec. at 92°C
		30 sec. at 57°C
		1 min. at 72°C
		For 22 cycles
	Followed by:	10 min. at 72°C

15µl of each PCR sample is separated on a 1.5% agarose gel. The Pharmacia 100bp ladder is used as a MW marker.

Mendelian segregation analysis

In order to demonstrate the Mendelian inheritance of the hemizygous transformation event CBH-351, the parental line was crossed with the non-transgenic line A619. The starting material is detasseled CBH-351, and fully fertile non-transgenic hybrid line A619. Upon crossing with the non-transgenic hybrid, offspring of the CBH-351 line will be tested for glufosinate tolerance. ECB resistance of glufosinate resistant plants will be evaluated by insect infestation. In this case, due to the hemizygous nature of transformation event CBH-351, a single insertion of transforming DNA will segregate at a 50/50 ratio, resulting in 50 % glufosinate tolerant plants.

Experimental design

Nine plots of about 250 transgenic corn plants CBH-351xA619 were planted, surrounded by 3 meters of non-transgenic corn plants. The number of germinating plants was recorded as indicated in table 1 below. At the 3-4 leaf stage, a glufosinate treatment was performed [Basta^R 3L/ha (600g ai/ha)]. Surviving plants were recorded as indicated in table 1 below. As co-integration of both transgenes is common, we have analyzed co-segregation of the transgenes by infesting 10-15 glufosinate-treatment surviving plants of three independent plots with ECB larvae. Leaf damage and/or stalk tunneling was scored on infested plants.
Statistical X^2-analysis were performed in order to confirm the confidentiality range.

III. Results

Southern analysis of transformation event CBH-351

A list of restriction enzymes and the expected size of digested DNA from CBH-351 corn plants, when probed with specific sequences, is presented in Table 1. The last page of Table 1, pg. 18, describes the actual plasmid location of each probe, when DNA from CBH-351 corn plants was isolated and digested with restriction enzyme(s), used to characterize the inserted DNA. The respective digests were analyzed by Southern blotting same blot with a DNA fragment comprising either the *bar* gene, the *cry9C* gene, the complete *P35S*, the *5' part of 35S* (pDE110 specific), the *P35 S*, the *bla* gene, a DNA fragment comprising the ori, or the complete plasmid pRVA9906. A representative example of the Southern blot analyses are shown in Figure 1. A plasmid map for pRVA9906, containing all the elements of pRVA9909 and pDE110, can be found in Figure 2.

Interpretation
A: In this hybridization, a fragment of the *cry9C* gene was used as probe. Due to the fact that a single hybridizing band is being observed in the SspI digest, it can be concluded that only one copy of the *cry9C* gene is present in transformation event CBH-351. The EcoRI and the combinational EcoRI/SspI confirm that a single intact copy of the *cry9C* gene is present.

B: In this hybridization, a fragment of the *bar* gene was used as a probe. All three digests indicate that 4 copies of the bar gene are present in transformation event CBH-351.

C: In this hybridization, the complete *P35S* (non-discriminating) was used as a probe. As the expression of both the *cry9C* gene and the *bar* gene is controlled by this promoter, an additive combination of all fragments retrieved in A and B should be observed. Indeed, careful comparison of the respective digests clearly indicate the combined pattern, indicating that the *35S* gene promoter is linked to each of the inserted *cry9C* and *bar* genes, except for the *bar* gene in the truncated pDE110 plasmid (see below).

D: In this hybridization, the complete plasmid pRVA9906, which comprises both the *cry9C* gene and the *bar* gene next to the same fragments of pUC19 as contained within the plasmids pRVA9909 and pDE110, was used as a probe. In this case, at least all hybridizing fragments observed in A, B, and C are expected. Careful comparison of the respective digests clearly

Table 1. Sizes of DNA fragments according to the probe and restriction enzyme

				PROBES			
DIGESTS	pRVA9906	*5' part P35S*	*P35 S*	*bar - 3'nos*	*bla*	*cry 9C*	*3' 35S*
EcoR I	14000						
	8250	8250	8250	8250			
	7000		7000	7000	7000	7000	
	4880	4880	4880	4880	4880		
	4600				4600		
	3200				3200		
	3100				3100		
	2250	2250	2250	2250			
	580						580
Hind III	18000		18000	18000	18000	18000	
	13600	13600	13600	13600	13600		
	13400	13400	13400	13400	13400		
	6400 (2x)	6400	6400	6400	6400 (2x)		6400
	1670					1670	1670
EcoR I - HindIII	8250	8250	8250	8250			
	5500		5500	5500	5500		
	3200				3200		
	3100				3100		
	2800				2800		
	2630				2630		
	2250	2250	2250	2250			2250
	1340					1340	
	330						330

Table 1. Sizes of DNA fragments according to the probe and restriction enzyme

DIGESTS	pRVA9906	5' part P35S	P35 S	PROBES bar - 3'nos	bla	cry 9C	3' 35S
SspI	12000				12000		
	10170	10170	10170	10170			
	8250	8250	8250	8250			
	5200		5200		5200	5200	5200
	4880	4880	4880	4880	4880		
	3000				3000		
	2550		2550	2550	2550		
EcoR I - Ssp I	8100	8100	8100	8100			
	(6000)	(6000)	6000	(6000)			
	4300	4300	4300	4300	4300		
	3000				3000		
	2550		2550	2550	2550	2550	
	2250	2250	2250	2250			
	2060				2060		
	580						580
Hind III - SspI	11000	11000	11000	11000	11000		
	4150	4150	4150	4150			
	2827	2827	2827	2827			
	2550		2550	2550	2550		
	2300				2300		2300
	2130				2130		
	2060				2060		
	1670					1670	1670
	1180		1180				

Table 1. Sizes of DNA fragments according to the probe and restriction enzyme

				PROBES			
DIGESTS	pRVA9906	*5' part P35S*	*P35 S*	*bar - 3'nos*	*bla*	*cry 9C*	*3' 35S*
EcoR V	> 20 Kb	> 20 Kb	> 20 Kb	> 20 Kb	> 20 Kb		
	13000	13000	13000	13000	13000		
	6250				6250	6250	6250
	2950		2950	2950	2950		
EcoR V - EcoR I	6400	6400	6400	6400			
	4880	4880	4880	4880	4880		
	4500				4500		
	3200				3200		
	2950		2950	2950	2950		
	2300	2300	2300	2300			
	1700					1700	
	580					580	580
EcoR V - Hind II	8500	8500	8500	8500	8500		
	6000	6000	6000	6000	6000		
	5700	5700	5700	5700	5700		
	4200				4200		4200
	2950		2950	2950	2950		
	1670					1670	1670
EcoR V - Ssp I	7400	7400	7400	7400			
	6700	6700	6700	6700			
	4880	4880	4880	4880	4880		
	4390				4390	4390	4390
	3000				3000		
	2830				2830		
	2000		2000	2000	2000		
Nco I	14000				14000	14000	14000
	9300	9300	9300		9300		
	6800			6800			
	6000	6000	6000	6000	6000		
	4880	4880	4880	4880	4880		
	2550		2550	2550	2550		

	PROBES		POSITION
pDE110 specific			
	5' part P35 S:	EcoRI - StuI fragment (550bp)	pDE110: from bp 397 until bp 947
	bar - 3' nos:	NcoI - HindIII fragment (866bp)	pDE110: from bp 1779 until bp 2645
pRVA9909 specific			
	cry9C:	SacI - NheI fragment (1942bp)	pRVA9909: from bp 11 until bp 1953
	3' 35S:	EcoRI fragment (578bp)	pRVA9909: from bp 1627 until bp 2205
Non-discriminating			
	bla:	MDB402 - VDS41 PCR fragment (866bp)	pDE110: from bp 3810 until bp 4676
			pRVA9909: from bp 3370 until bp 4236
	Complete P35S:	EcoRI - NcoII fragment (1382bp)	pDE110: from bp 397 until bp 1779
			pRVA9909: from bp 4621 until bp 5145
	P35 S:	BglII - NcoI fragment (996bp)	pDE110: from bp 783 until bp 1779
			pRVA9909: from bp 4621 until bp 5145
	pRVA9906:	Total plasmid, linearized with BamHI	all features of pDE110 and pRVA9909

indicate the combined pattern. The additional bands indicate the presence of pUC19 DNA sequences such as the origin of replication and the *bla* gene in transformation event CBH-351.

A detailed description of the presence of the *bla* gene linked to the ori sequence is given separately below.

Characterization of the inserted DNA in event CBH-351

A series of Southern blot analyses were performed to characterize the inserted DNA for ***Bt*** Cry9C corn event, CBH-351. Each insert is explained separately below. Refer to Figure 3. Schematic drawing of the '*cry9C* insert' and Figure 4. Schematic of the pDE110 insert. Features of the HindIII fingerprint is summarized in Table 2 (page 20).

'cry9C insert'

- 18000 bp HindIII fragment: This fragment hybridizes with the *bla, bar-3'nos, cry9C* and the *P35 S* probes. It doesn't hybridize with the *5' part P35S* probe (pDE110 specific probe). This HindIII fragment carries a junction between the pRVA9909 plasmid and part of a pDE110 plasmid. Most probably there are no, or very little, *P35S* sequences of the pDE110 plasmid integrated with the *bar-3'nos* sequences in this insert - we don't observe hybridization with the *5' part P35S* and when probing with the *P35 S* probe, the obtained hybridization signal doesn't indicate the presence of two *P35S* copies. The HindIII recognition site is at least 13 Kb upstream of this insert.

- 6400 bp HindIII fragments: One of the 6400 bp HindIII fragments hybridizes with the *3' 35S* and *bla* probes. This 6400 bp HindIII fragment is part of the *cry9C* insert. A 14000 bp NcoI fragment hybridizes with the *cry9C, 3' 35S* and *bla* probes. It doesn't hybridize with any of the pDE110 specific probes. This NcoI fragment carries the 1700 bp HindIII fragment (cry9C) and the 6400 bp HindIII fragment (*3' 35S* and *bla*). This indicates that there is at least 9000 bp of plant DNA present on this fragment.

- 1700 bp HindIII fragment: This fragment hybridizes only with the *cry9C* probes since it is the internal *cry9C* HindIII fragment.

The '*cry9C* insert' contains three co-linear integrated HindIII fragments - a 18000 bp, a 6400 bp and a 1700 bp HindIII fragment. This inserted copy carries a small part of the pDE110 plasmid (*bar-3'nos* sequences) and the pRVA9909 plasmid. There are at least 13000 bp of plant DNA between the upstream HindIII site and the insert. This insert is flanked downstream by at least 9000 bp of plant DNA.

pDE110 insert

- 2 copies of pDE110, Head-to-Tail insert: With EcoRI digested CBH-351 genomic DNA a 4880 bp fragment is observed that hybridized to every feature of pDE110. A fragment of similar size was also found when the genomic DNA was digested with restriction enzymes

having one restriction site in the pDE110 plasmid (SspI, NcoI, EcoRI, or BamHI). These fragments are indicative for a Head-to-Tail insertion of two pDE110 plasmid copies.

The Head-to-Tail insertion was confirmed by means of PCR, using one primer upstream of the pDE110 HindIII site (restriction site used to linearize the plasmid before transformation) and one primer downstream of the HindIII site. The amplified fragment had the expected size and carries a HindIII restriction site.

- 3rd copy of pDE110: A third complete copy of the pDE110 plasmid is inserted separately.

The Head-to-Tail pDE110 and the pDE110 inserts are located on three HindIII fragments: a 13.6 Kb, a 13.2Kb and a 6400 bp HindIII fragment. The extensive Southern blot characterization conducted so far doesn't allow us to determine the respective organization of the inserted DNA fragments relative to each other. Pulse field electrophoresis and/or cloning of the CBH-351 transformation locus will allow us to determine the organization of the CBH-351 transformation locus.

Table 2. Features of the HindIII Fingerprint - pRVA9906 probe

HindIII fragments	Features
18 Kb	Part of the Cry9C insert: - P35S - bar-3'nos - bla - 5' cry9C
13.6 Kb	pDE110: - 5' part P35 S - P35 S - bar-3'nos - bla
13.2 Kb	pDE110: - 5' part P35 S - P35 S - bar-3'nos - bla
6400 bp	Part of the Cry9C insert: - 3' 35S - bla
6400 bp	pDE110: - 5' part P35 S - P35 S - bar-3'nos - bla
1700 bp	Part of the Cry9C insert: - cry9C

Conclusion: In transformation event "CBH351", one copy of the cry9C gene and 4 copies of the bar gene are present. All gene copies, except one, are flanked by the 5' promoter of the 35S gene, indicating that the inserted traits are to be expressed in most plant tissues. The DNA insertion comprises three fragments which include a single copy of the pDE110 plasmid, a head to tail linked double copy of the pDE110 plasmid and a combined copy of a truncated pDE110 plasmid linked to the pRVA9909 plasmid.

Determination of the *bla* gene presence in corn transformation event CBH-351

The presence of the *bla* gene was verified by PCR analysis and southern blotting.

Using 3 different sets of primers, the presence of the beta-lactamase secretion signal, the active site and the substrate binding site was verified. The position of the respective primers is shown in Figure 2. All three sets of primers yielded a PCR DNA fragment of the correct size, as predicted by the known DNA sequence (data not shown).

Primer	Sequence (5'-3')
VDS47	CAT CGA ACT GGA TCT CAA CAG C
VDS44	TGT CAT GCC ATC CGT AAG ATG C
AMPOL6	CGG TCG CCG CAT ACA CTA TTC TCA G
AMPOL5	CTG CGT TTG GTA TGG CTT CAT TCA G
AMPOL2	GAG GCG GAT AAA GTT GCA GGA CCA
AMPOL1	CAG TGA GGC ACC TAT CTC AGC G

Southern analysis using a *bla* gene probe confirmed these results (see Figure 6). DNA of corn CBH-351 was extracted and digested with the restriction enzymes BspHI. After hybridization with the *bla* gene probe, positive signals were obtained, indicating that the bacterial *bla* gene is present in corn transformation event CBH-351.

Conclusion: The bacterial bla gene is present in corn transformation event CBH-351.

Cry9C and PAT expression at mRNA level

In order to demonstrate the expression of the *cry9C* in various organs of transformation event CBH-351, Northern analysis using a probe transcribed from the *cry9C* or the *bar* gene has been performed (respectively, Figure 7 and Figure 8). To ensure that the RNA was not degraded in the sample, visual observations of the ribosomal RNA bands were made of the original gel. If degradation of the RNA occurred, the gel was discarded.

Interpretation

- Expression of the *cry9C* gene: the *cry9C* gene is expressed in all organs tested (leaf, stem, root, ear and tassel) at varying levels (ranging between 5 to about 60 pg per 5ug mRNA) with the exception of seed. Evidently the level in seeds is below the limit of detection.

- Expression of the *bar* gene: the *bar* gene is expressed in all organs tested (leaf, stem, root, ear and tassel) at varying levels (ranging between 5 to about 60 pg per 5ug mRNA) with the exception of seed. Evidently the level in seeds is below the limit of detection.

Conclusion: the cry9C and the bar gene are expressed in all organs tested (leaf, stem, root, ear and tassel) at varying levels. These data indicate that at least one functional 5' promoter of the 35S gene is linked to this gene in transformation event CBH-351, confirming the data obtained by Southern analysis (see above).

Evaluation of cryptic expression of the *bla* gene

In transformation event CBH-351, the *bla* gene is flanked by bacterial transcription regulatory sequences. Expression in plants is unlikely. Nevertheless, unintended expression of the corresponding DNA sequence e.g. as a result of read-through phenomena, could lead to the unexpected expression of the *bla* gene. Such expression is often described as 'cryptic expression'.

In order to determine whether the *bla* gene present in transformation event CBH-351 is unexpectedly expressed, northern analysis has been performed on different tissues. As a probe, radioactively labeled T7 transcript of VDS40-VDS41 was used. The results of this analysis is shown in Figure 9.

No positive signal could be detected in leaf, root, stem or seed, indicating that the *bla* gene is not expressed in any of these tissues.

Conclusion: The bla gene present in transformation event CBH-351 is not expressed in the corn plant tissues.

Stability of the insert in different genetic backgrounds

Different crosses of corn plants derived from event CBH-351 were analyzed by Southern analysis in order to determine the stable conservation of the transferred DNA across different genetic backgrounds.

DNA was isolated according to Dellaporta *et al.* (1983). The DNA of the hybrid plants was digested by the restriction enzyme HindIII. The whole plasmid pRVA9906, containing both the *cry9C* gene and the *bar* gene was linearized with BamHI and used as a probe. An example of Southern analysis results is given in Figure 10.

The hybridization data revealed that all the analyzed offspring had an identical integration pattern as the primary transformation event CBH-351.

Conclusion: The CBH-351 allele is stably inherited in different genetic backgrounds.

Stability of the insert in different generations

Five different crosses of corn plants derived from event CBH-351 were analyzed by Southern analysis in order to determine the stable conservation of the transferred DNA during multiple generations.

DNA was isolated according to Dellaporta *et al.* (1983). The DNA of the hybrid plants was digested by the restriction enzyme HindIII. The whole plasmid pRVA9906, containing both the *cry9C* gene and the *bar* gene was linearized with BamHI and used as a probe. The initial transformant (designated "To" in the figure) was crossed sequentially to H99 in a series of backcrosses to produce 5 different generations. An example of Southern analysis results is given in Figure 11.

The hybridization data revealed that all the analyzed offspring had an identical integration pattern as the primary transformation event CBH-351.

Conclusion: The CBH-351 allele is stably inherited over 5 generations.

Analysis of Mendelian segregation of transformation event CBH-351

In order to demonstrate the stability of the hemizygous transformation event CBH-351, Mendelian inheritance was performed on the offspring of a CBH-351 parental corn line and a non-transgenic corn line. A single insertion of transforming DNA will segregate at a 50/50 ratio, resulting in 50% glufosinate tolerant plants.

Field Analysis

Table 3. Overview of the field experimentation with the corn plants CBH-351xA619

Plot #	# of Plants	Basta Sensitive	Basta Resistant	Sen./Tol. Expected	X^2 value
1	239	135	104	119.5	3.766
2	269	123	146	134.5	1.799
3	247	139	108	123.5	3.644
4	261	124	137	130.5	0.552
5	256	140	116	128	2.066
6	261	143	118	130.5	2.207
7	224	117	107	112	0.361
8	220	115	105	110	0.368
9	211	108	103	105.5	0.076
Total	2188	1144	1044	1094	4.479

In plots 1, 5, and 9 respectively 10 glufosinate-resistant plants were infested with ECB to score leaf damage and 15 glufosinate-resistant plants were infested with ECB to score stalk tunneling. In all cases, no damage of the infested plants was observed.

Interpretation
The field analysis of the CBH-351xA619 corn plants indicates that the transformation event CBH-351 segregates at a 1:1 ratio. Moreover, the ECB infestation data clearly indicate the co-segregation of the glufosinate-resistance and ECB-tolerance traits.

Conclusion: The corn transformation event CBH-351 represents a single genetic locus comprising both the glufosinate resistance trait (the bar gene) and the insecticidal resistance trait (the cry9C gene)

Names of Key Individuals in Study

Name	Title
Johan Botterman	Laboratory Supervisor
Marc De Beuckeleer	Researcher
Veronique Gossele	Junior Researcher
Stefan Jansens	Senior Researcher
Jan Janssens	Researcher
Nele Vandermarliere	Laboratory Assistant

Disposition of Raw Data

All original study data generated from this study will be archived along with the final version of this report at Plant Genetic Systems N.V.

References

Southern, E.M., *J. Mol. Biol.*, **98:** 503-517, 1975.
Sambrook, J, Fritsch, E.F., and Maniatis, T., In: *Molecular Cloning, Second Ed.*, (Ed. Sambrook, J.), pp 9.42-9.55, 1989.
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K. Edwards *et al.*, NAR vol 19, No 6, page 1349, 1991).
Jones J.D.G., Dunsmuir P. and Bedbrook J., *The EMBO Journal*, 4, 2411-2418, 1985.
White, T.J., Arnheim, N., Erlich, H.A., *TIG*, **5:** 185-189, 1989.

Legend to the figures

Figure 1:
Southern hybridization of DNA from the corn transformation event CBH-351. The gel was probed with radioactive labeled DNA fragments as indicated in A-D below. Plant DNA was digested with the restriction enzyme EcoRI (E), SspI (S), and both EcoRI and SspI (ES). Molecular weight markers are indicated at the right.

1A. Probe: *cry9C* gene
1B. Probe: *bar* gene.
1C. Probe: Complete *P35S*
1D. Probe: plasmid pRVA9906 DNA, linearized with the restriction enzyme BamHI

Figure 2:
Plasmid map of pRVA9906.

Figure 3:
Schematic drawing of the '*cry9C* insert'.

Figure 4:
Schematic drawing of the pDE110 insert.

Figure 5:
Schematic representation of the *bla* gene with indication of the applied primer sets.

Figure 6:
Southern blot analysis of total DNA from the corn transformation events CBH-351(lane 1) and CBH-354 (lane 2). A radioactively labeled DNA fragment containing the *bla* gene was used as a probe. A 100 bp molecular weight ladder was used in lane 3.

Figure 7:
Northern analysis of total RNA fractions of different tissues of corn transformation event CBH-351: evaluating the expression of the *cry9C* gene. This blot was probed with a DNA fragment comprising the *cry9C* gene. Molecular weight markers are indicated at the left. (lane 2,3,4: leaf tissue; lane 5,6,7: root tissue; lane 8,9,10: stem tissue; lane 11,12: seeds; lane 13- 18: calibration RNA series of *in vitro* produced *cry9C* mRNA respectively 5pg, 10 pg, 20 pg, 40pg, 80 pg, and 160 pg mRNA; in lanes 2-12, 5 ug of total mRNA was loaded).

Figure 8:
Northern analysis of total RNA fractions of different tissues of corn transformation event CBH-351: evaluating the expression of the *bar* gene. This blot was probed with a DNA fragment comprising the *bar* gene. Molecular weight markers are indicated at the left. (lane 2,3,4: leaf tissue; lane 5,6,7: root tissue; lane 8,9,10: stem tissue; lane 11,12: seeds; lane 13- 18: calibration RNA series of *in vitro* produced *bar* mRNA respectively 10 pg, 20 pg, 40pg, 80 pg, 160 pg, and 320 pg mRNA; in lanes 2-12, 5 ug of total mRNA was loaded).

Figure 9:
Northern analysis of total RNA fractions of different tissues of corn transformation event CBH-351: evaluating the expression of the *bla* gene. This blot was probed with a DNA fragment comprising the *bla* gene transcript. (Lane 1&13: MW marker (G319, promega); lane 2,3,4: leaf tissue; lane 5,6,7: root tissue; lane 8,9,10: stem tissue; lane 11,12: seed; lane 14-18: dilution series of *bla* gene VDS40-VDS41 SP6 transcript (0.5pg, 1pg, 2pg, 4pg, and 8pg).

Figure 10:
Southern Blot Analysis - Stability of the insert of CBH351 in different backgrounds
Genetic stability of transformation event CBH-351 across different genetic backgrounds. Southern hybridization of DNA from the respective crosses with corn transformation event CBH-351 probed with radioactive labeled plasmid pRVA9906 DNA, linearized with the restriction enzyme BamHI. Plant DNA was digested with the restriction enzyme HindIII. Molecular weight markers are indicated at the left. Lanes:1. (((B73 x (To x B73)) x Mo17); 2. (Mo17 x ((B73 x (To x B73)).x B73)); 3. (A619 x ((B73 x (To x B73)) x B73)); 4. (B73 x ((B73 x (To x B73)) x B73)); 5. B73 wild type; 6. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

Figure 11:
Southern Blot Analysis - Stability of the insert of CBH351 in different generations. Genetic stability of transformation event CBH-351 in multiple crosses. Southern hybridization of DNA from the respective crosses with corn transformation event CBH-351 probed with radioactive labeled plasmid pRVA9906 DNA, linearized with the restriction enzyme BamHI. Plant DNA was digested with the restriction enzyme HindIII. Molecular weight markers are indicated at the left. Lanes:1. T1 (H99 x To); 2. T2 (H99^2 x To); 3. T3 (H99^3 x To); 4. T4 (H99^4 x To); 5. T5 (H99^5 x To); 6. B73 wild type. 7. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

Figure 1

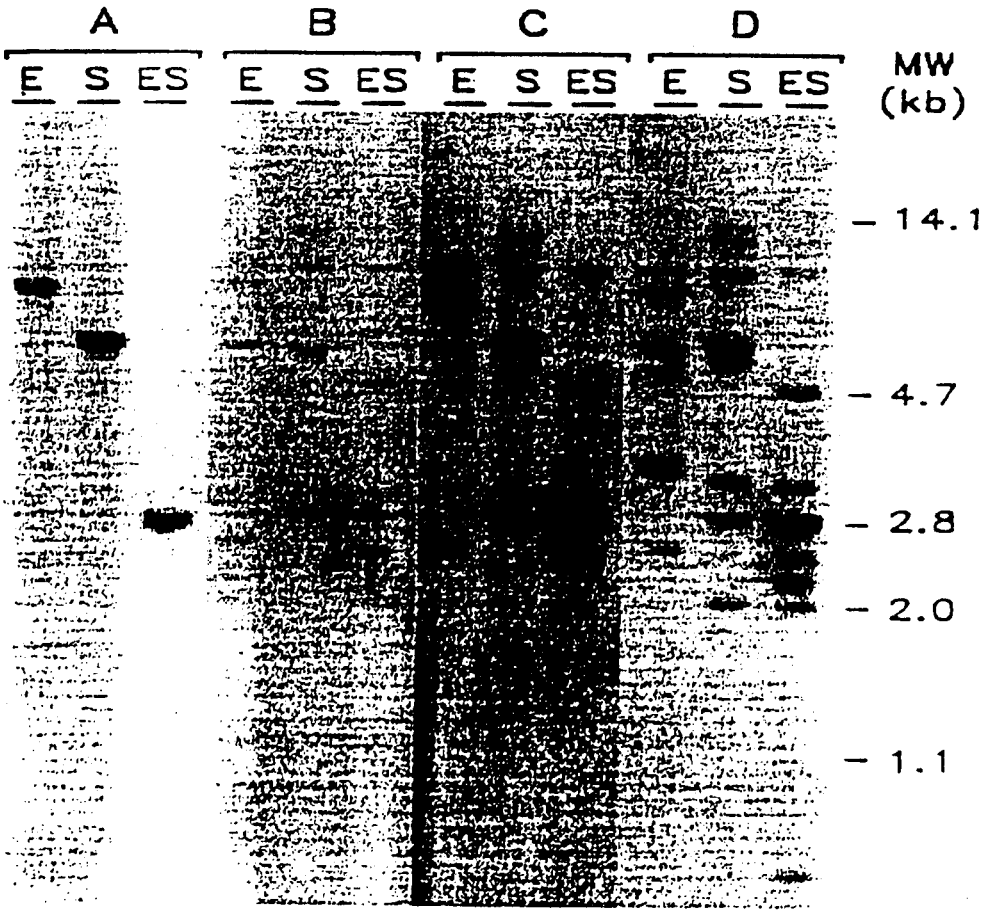
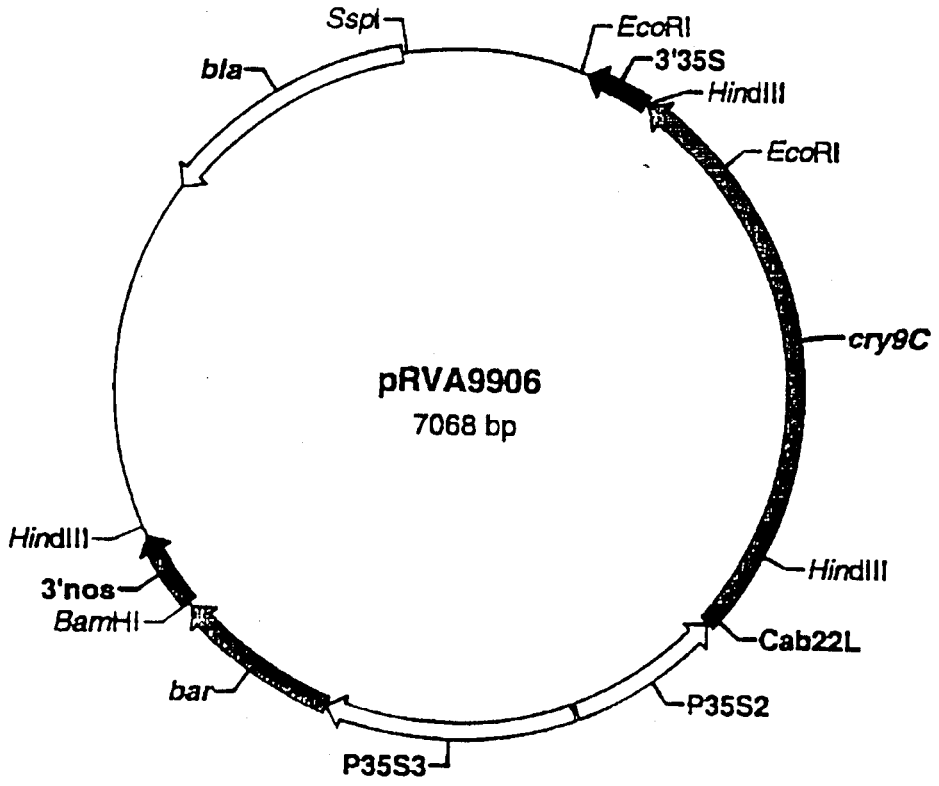


Figure 2. Plasmid map of pRVA9906



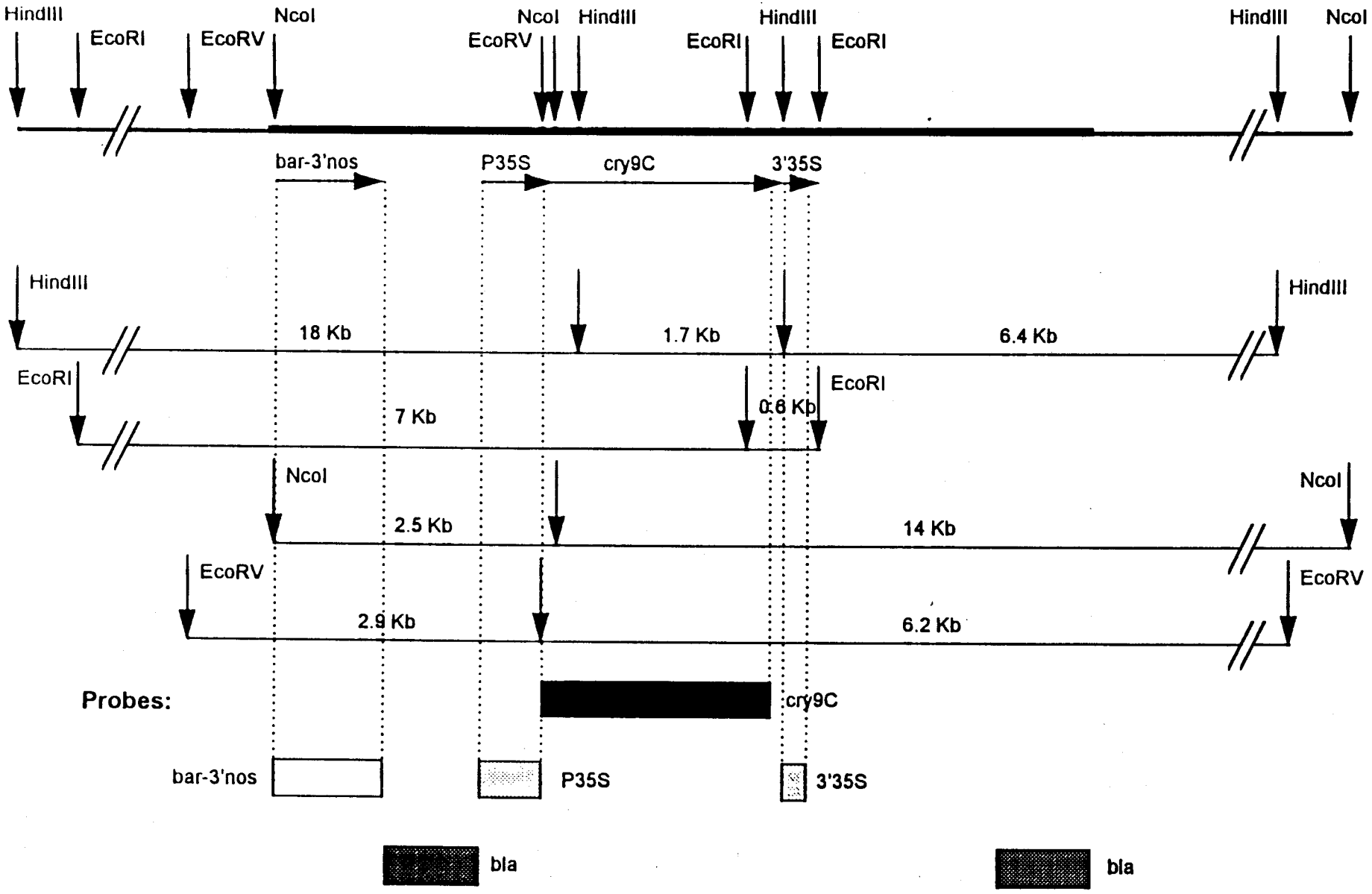
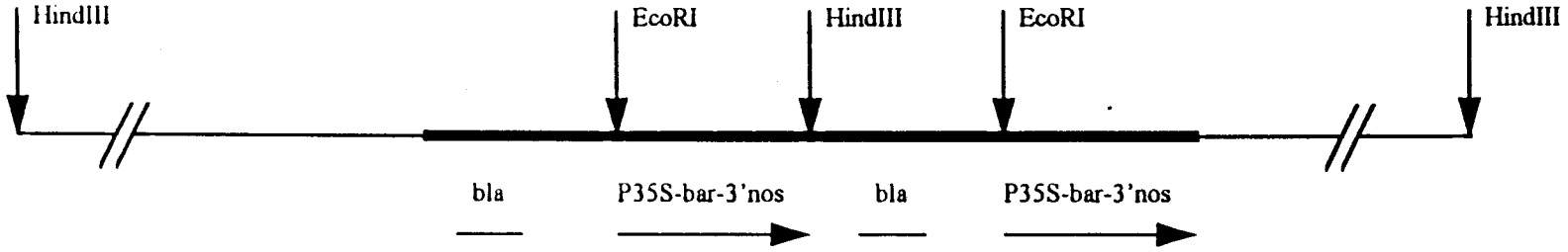
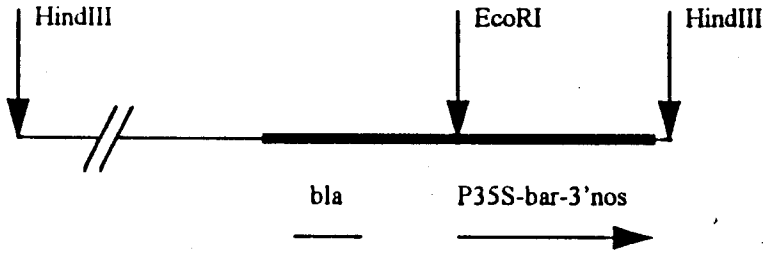


Figure 3. Schematic drawing of the '*cry9C* insert'. Each line represents a specific restriction digest.



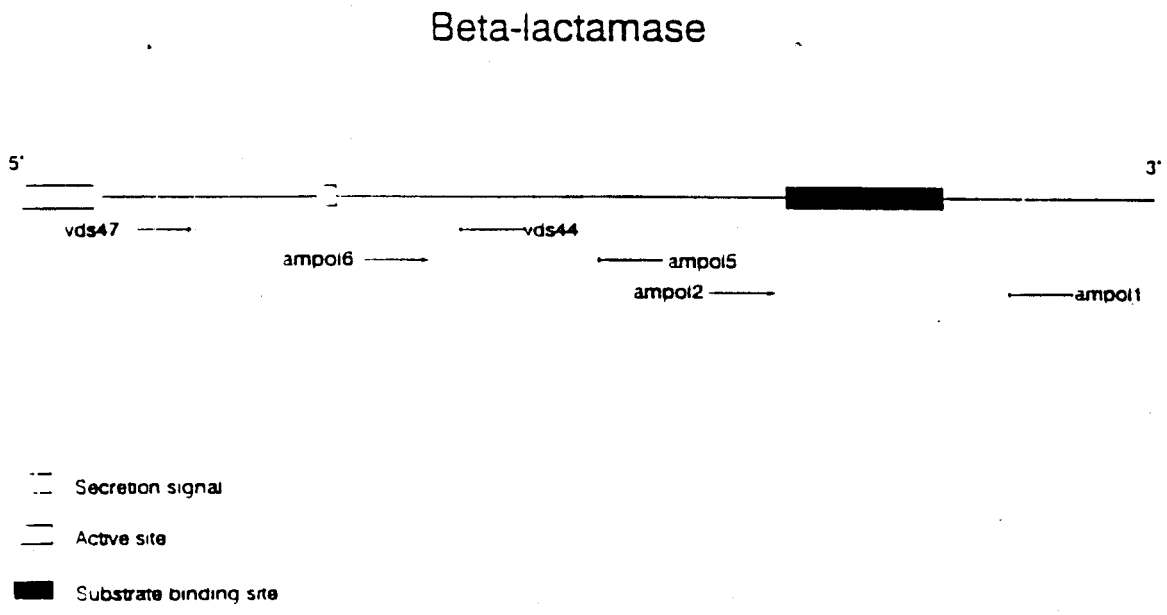
Schematic drawing of the Head-to-Tail pDE110 insert



Schematic drawing of the pDE110 insert

Figure 4. Schematic drawings of the pDE110 inserts

Figure 5


Beta-lactamase
5'
3'
vds47
vds44
ampol6
ampol5
ampol2
ampol1
Secretion signal
Active site
Substrate binding site

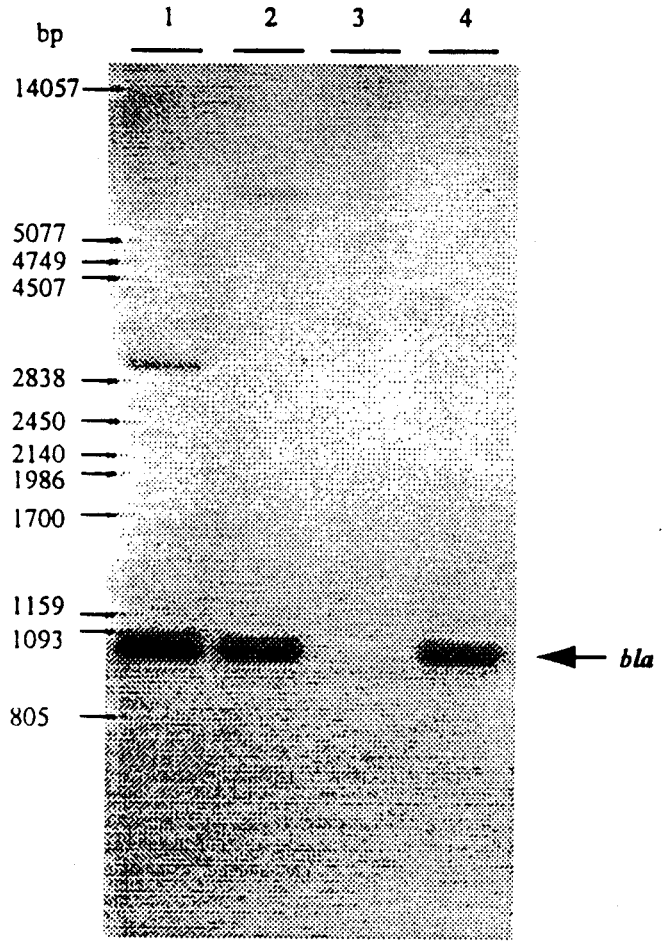


Figure 6: Southern Blot Analysis - presence of the *bla* gene
Probe: *bla* gene. Digest: BspHI
1. CBH351
2. CBH354
3. B73 wild type
4. B73 wild type + 3 copies pDE110-BspHI

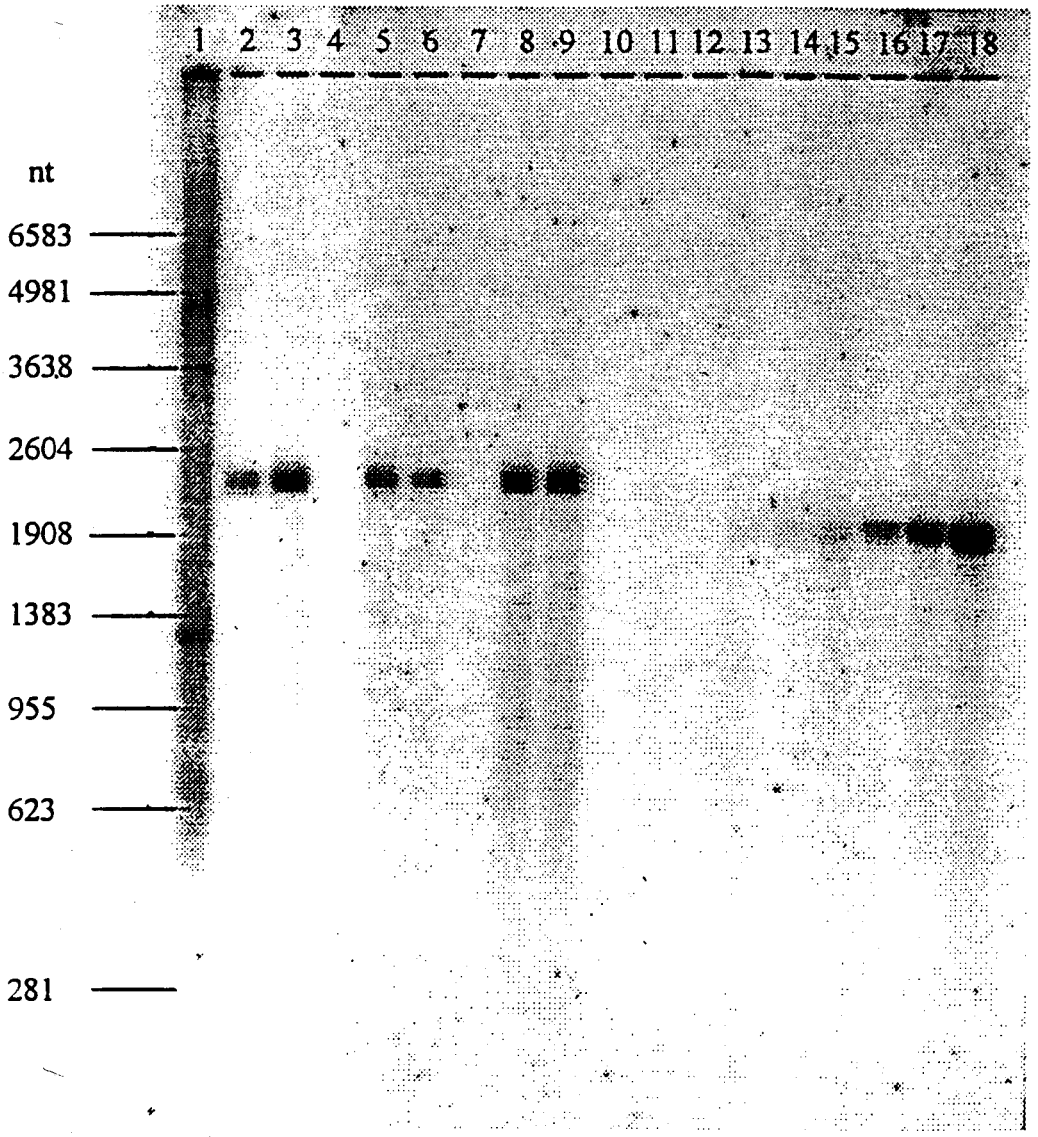


Figure 7: Northern Blot Analysis CBH351 - anti-sense *cry9C* probe
1. MW Marker; 2. leaf RNA plant A; 3. leaf RNA plant B; 4. B73 wild-type leaf RNA; 5. root RNA plant A; 6 root RNA plant B; 7. B73 wild-type root RNA; 8. stem RNA plant A; 9. stem RNA plant B; 10. B73 wild-type stem RNA. 11. dry seeds RNA (CBH-351, lot # 96ZM001879); 12. B73 wild type dry seeds RNA; 13.-18. calibration RNA series of *in vitro* produced sense *cry9C* RNA - respectively 5, 10, 20, 40, 80 and 160 pg.

ANNEX 6
Bt Cry9C Corn

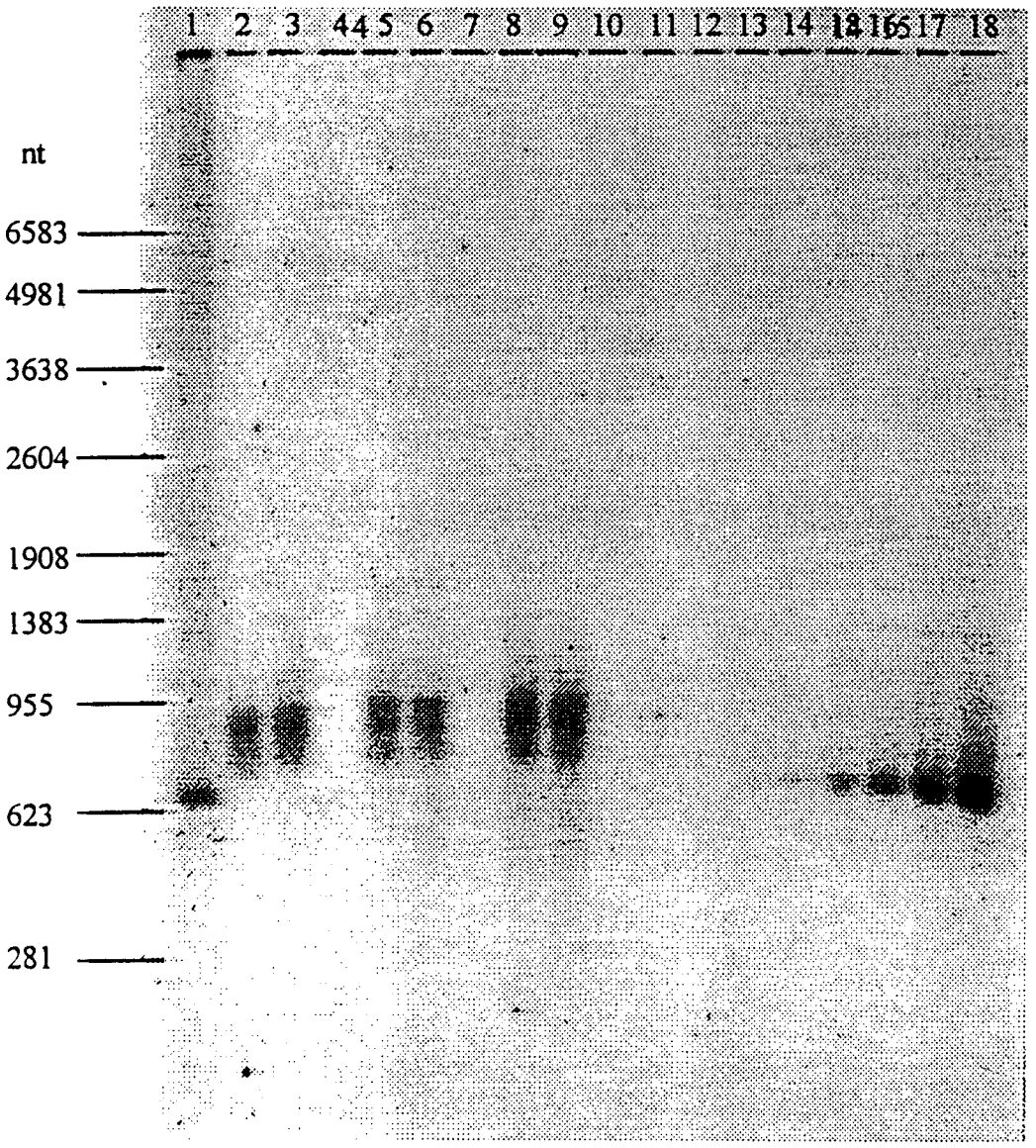


Figure 8: Northern Blot Analysis CBH-351 - anti-sense *bar* probe
1. MW Marker; 2. leaf RNA plant A; 3. leaf RNA plant B; 4. B73 wild type leaf RNA; 5. root RNA plant A; 6. root RNA plant B; 7. B73 wild type root RNA; 8. stem RNA plant A; 9. stem RNA plant B; 10. B73 wild type stem RNA; 11. dry seeds RNA (CBH-351, 96ZM001879); 12. B73 wild type dry seeds RNA; 13.-18. calibration RNA series of *in vitro* produced sense *bar* RNA - respectively 10, 20, 40, 80, 160 and 320 pg.

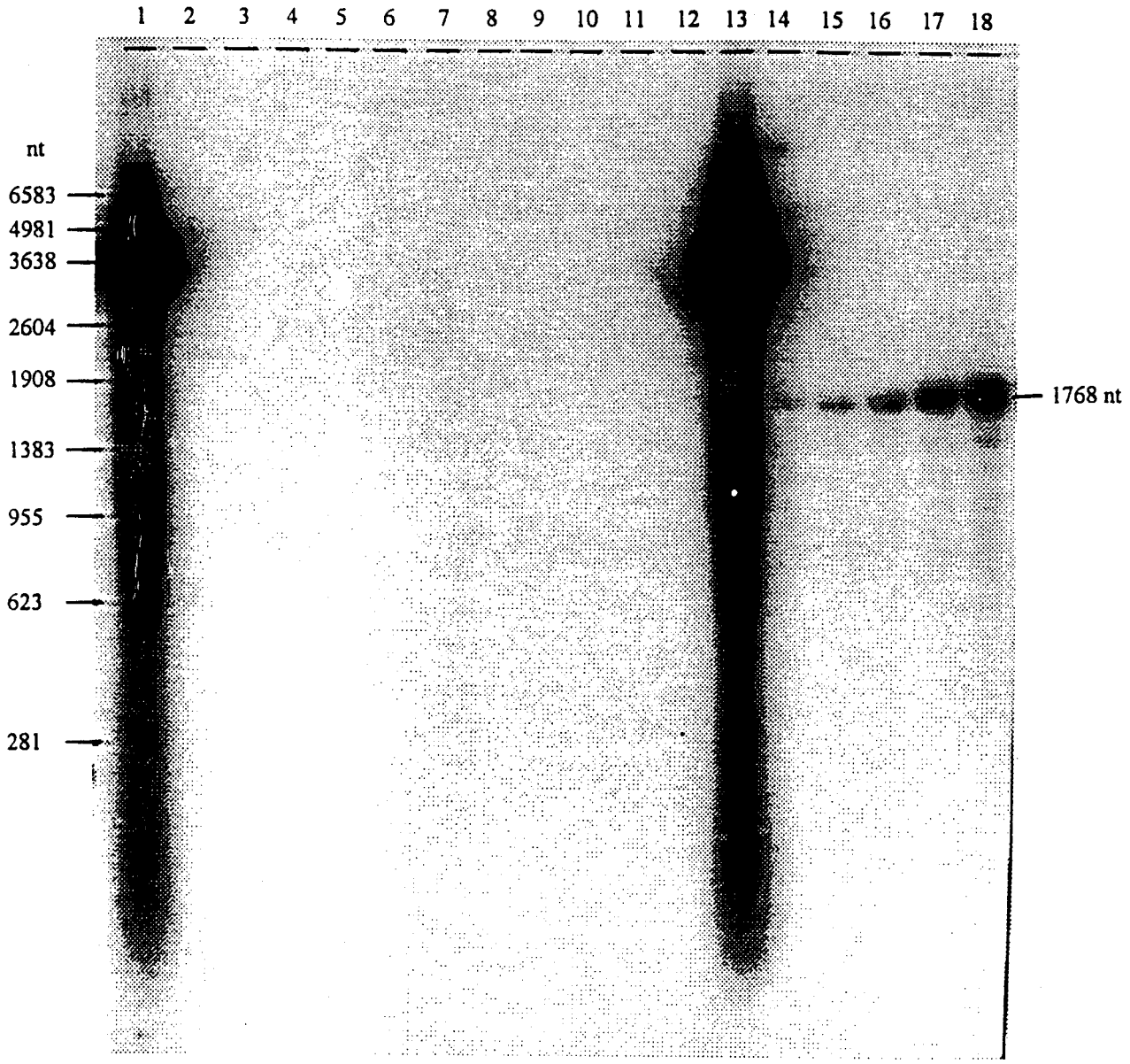


Figure 9: Northern Blot Analysis CBH351 - Evaluation of cryptic expression of the *bla* gene
Probe: anti-sense *bla* + *ori*
1. MW marker; 2. leaf RNA plant A; 3. leaf RNA plant B; 4. leaf RNA wild type control; 5. root RNA plant A; 6. root RNA plant B; 7. root RNA wild type control; 8. stem RNA plant A; 9. stem RNA plant B; 10. stem RNA wild type control; 11. dry seeds RNA (CBH-351, lot #96ZM001879); 12. dry seeds RNA wild type control; 13. MW marker; 14. - 18. calibration series of *in vitro* produced sense *bla* + *ori* RNA respectively 0.5, 1, 2, 4 and 8 pg.

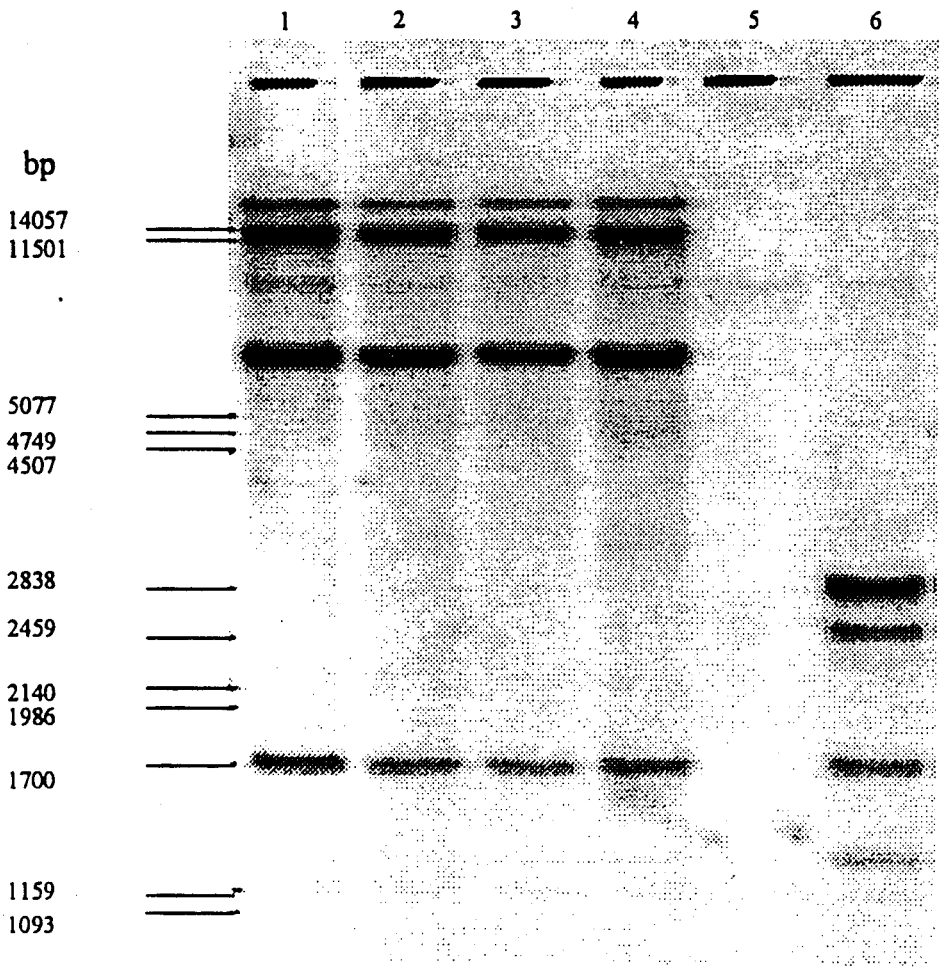


Figure 10: Southern Blot Analysis - Stability of the insert of CBH351 in different backgrounds

Genomic DNA digested with HindIII and probed with pRVA9905

1. (((B73 x (To x B73)) x Mo17);
2. (Mo17 x ((B73 x (To x B73)) x B73));
3. (A619 x ((B73 x (To x B73)) x B73));
4. (B73 x ((B73 x (To x B73)) x B73));
5. B73 wild type;
6. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

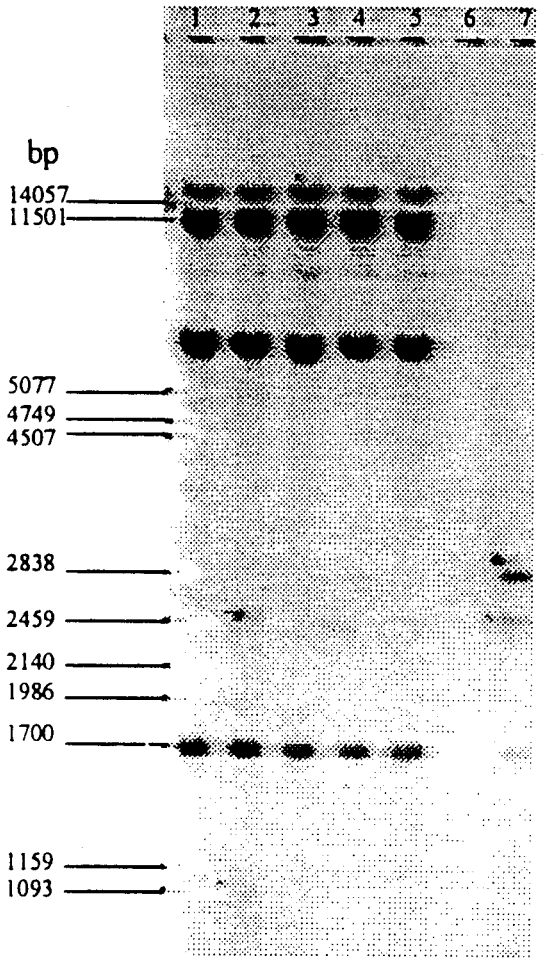


Figure 11: **Southern Blot Analysis - Stability of the insert of CBH351 in different generations**
Genomic DNA digested with HindIII and probed with pRVA9906 1. T1 (H99 x To). 2. T2 (H99^2 x To). 3. T3 (H99^3 x To). 4. T4 (H99^4 x To). 5. T5 (H99^5 x To). 5. B73 wild type. 6. B73 wild type, supplemented with one copy of pRVA9906 - HindIII digested.

ANNEX 7
Bt Cry9C Corn

IOWA STATE UNIVERSITY
OF SCIENCE AND TECHNOLOGY

Department of Entomology
Insectary Building
Ames, Iowa 50011-3140
515 294-7400
FAX 515 294-8027

23 January 1997

Ms. Susan C. MacIntosh
Regulatory Affairs Manager
Plant Genetic Systems (America) Inc.
7200 Hickman Rd, Suite 202
Des Moines, IA 50322

Dear Susan:

None of the following insects are pests of corn.

- Beetle, American burying (*Nicrophorus americanus*)
- Beetle, Coffin Cave mold (*Batrisodes texanus*)
- Beetle, delta green ground (*Elaphrus viridis*)
- Beetle, Hungerford's crawling water (*Brychius hungerfordi*)
- Beetle, Kretschmarr Cave mold (*Texamaurops reddelli*)
- Beetle, northeastern beach tiger (*Cicindela dorsalis dorsalis*)
- Beetle, Puritan tiger (*Cicindela puritana*)
- Beetle, Tooth Cave ground (*Rhadine persephone*)
- Beetle, valley elderberry longhorn (*Desmocerus californicus dimorphus*)
- Butterfly, bay checkerspot (*Euphydryas editha bayensis*)
- Butterfly, El Segundo blue (*Euphilotes battoides allyni*)
- Butterfly, Karner blue (*Lycaeides melissa samuelis*)
- Butterfly, Lange's metalmark (*Apodemia mormo langei*)
- Butterfly, lotis blue (*Lycaeides argyrognomon lotis*)
- Butterfly, mission blue (*Icaricia icarioides missionensis*)
- Butterfly, Mitchell's satyr (*Neonympha mitchellii mitchellii*)
- Butterfly, Myrtle's silverspot (*Speyeria zerene myrtleae*)
- Butterfly, Oregon silverspot (*Speyeria zerene hippolyta*)
- Butterfly, Palos Verdes blue (*Glaucopsyche lygdamus palosverdesensis*)
- Butterfly, Saint Francis' satyr (*Neonympha mitchellii francisci*)
- Butterfly, San Bruno elfin (*Callophrys mossii bayensis*)
- Butterfly, Schaus swallowtail (*Heraclides aristodemus ponceanus*)
- Butterfly, Smith's blue (*Euphilotes enoptes smithi*)
- Butterfly, Uncompahgre fritillary (*Boloria acrocnema*)
- Dragonfly, Hine's emerald (*Somatochlora hineana*)
- Fly, Delhi Sands flower-loving (*Rhaphiomidas terminatus abdominalis*)
- Moth, Kern primrose sphinx (*Euproserpinus euterpe*)
- Naucorid, Ash Meadows (*Ambrysus amargosus*)
- Skipper, Pawnee montane (*Hesperia leonardus (=pawnee) montana*)

Sincerely,

Marlin E. Rice
Professor of Entomology
Extension & Research

HOLDEN'S FOUNDATION SEEDS, INC.
P.O. Box 839, Williamsburg, Iowa 52361
FAX 319 668-2453
319 668-1100

2 May 1997

Field Trial Report
USDA Notification Number: 94-339-05N
(applicant reference: PGS BT R)

Submitted by: Dr. Lori Marshall
Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, Iowa 52361
phone: 319/668-1100
fax: 319/668-2453

Test site information:
The 2 plantings were grown from January 1995 to September 1995, in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai.

Summary of experimental results:
Segregation. The plants segregated as expected for a single nuclear gene when treated with glufosinate. No insects were used to evaluate insect resistance, and standard insecticides were used to control insect pests, so there was no information obtained on the transgenic plants' response to insects. The transgenic plants are in a backcrossing program in which the Bt genes are being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and the selectable marker gene for glufosinate tolerance appeared to segregate and express as expected in this wide sampling of genetic backgrounds.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the

course of the growing season the following circumstances allowed comparision of transgenic vs non-transgenic corn lines:

Response to standard irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Flowering - no differences detected.

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected.

The transgenic plants displayed the expected tolerance to glufosinate but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

This transgenic material was planted 2 times during the period from January 1995 to September 1995. For both plantings, isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

January 18, 1995 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.1 acre.

May 22, 1995 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.25 acre.

Termination of exeriment

January 18, 1995 planting: Hand-pollinated ears were harvested from t trial May 2, 1995, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early May 1994.

May 22, 1995 planting: Hand-pollinated ears were harvested from the tr August 30, 1995, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early September, 1995.

For all plantings: After this initial disking into the soil, the sites were left fallow for several months. After the initial disking each site went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Monitoring for Volunteers

For all plantings: During the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings i Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. Durin the third cycle, no volunteers were observed, as is typical in our standa plantings.

I. DEKALB FIELD RESULTS 1995 FOR CBH-351

REGULATORY INFORMATION

1. *APHIS Notification Number:* 95-087-05N for both a & b
2. *County and State of release:*
   a) Champaign Co., IL - efficacy
   b) Maui Co., HI - breeding
3. *Planting date:*
   a) 5/22/95
   b) 8/24/95 & 11/22/95
4. *Isolation method for gene containment:* Spatial (660' +) for both a & b
5. *Harvest date:*
   a) 10/16/95 (grain was harvested and burned on isolation site)
   b) 11/09-23/95 & 2/14-28/96 respectively

SUMMARIZING REMARKS

A) Efficacy

1. *Preparation of site and treatments:* General agronomics for corn in central IL including discing, field cultivation, incorporation of Dual and Broadstrike herbicides, planting using in-furrow Lorsban insecticide, rotary hoe, and one cultivation.
2. *Description of experiment:* RCB, 2 reps. All plants except nonBt controls were wiped (1-2" leaf band) with a 4% solution of Liberty (glufosinate) herbicide, susceptible plants were identified and removed. Remaining plants were artificially infested 5 times with ECB larvae at the rate of 30-50 per plant per infestation at mid whorl state (ca. V8). ECB1 leaf feeding damage was evaluation at 3 weeks post final infestation. All plants were further infested at flowering with ECB larvae 4 times at the rate of 30-50 per plant. ECB2 stalk damage was evaluated at 2 weeks before harvest by splitting stalks and examining tunneling.
3. *General results and observations:* Bt plants exhibited excellent control of both ECB1&2 as evidenced by greatly reduced (near 0) leaf feeding and stalk tunneling relative to nonBt entries.
4. No differences between transgenic and non-transgenic plants were observed except resistance to glufosinate and ECB.
5. *Disposal of plants and seeds:* All plants were shredded, seeds were collected, and all transgenic material was burned on the isolation site.
6. *Volunteers:* Monitored, removed, and burned in July 1996.

B) Breeding

1. *Preparation of site and treatments:* General agronomics for corn including discing, incorporation of simazine herbicide, planting using in-furrow Lorsban insecticide.
2. *Description of experiment:* Breeding triplets. All plants except nonBt recurrent parents sprayed with a 1X rate (28 oz/acre) of Liberty (glufosinate) herbicide, susceptible plants were identified and removed. Several of the remaining plants were cross pollinated.
3. *General results and observations:* Pollinations with Bt plants were successful.
4. No differences between transgenic and non-transgenic plants were observed except resistance to glufosinate.
5. *Disposal of plants and seeds:* Non-harvested plants and seeds were shredded and turned under the soil at the isolation site.
6. *Volunteers:* Monitored and destroyed on site within 30 days of harvest (after irrigation).

Asgrow Seed Company

Final Report to the USDA - August 6th, 1996

USDA Permit Number :	95-089-04N
Asgrow Permit Number:	ASG32795.B
Locations:	Isabela, Puerto Rico, Ames, Iowa - Story County Gibbon, Nebraska - Buffalo County
Responsible Researchers:	Dr. Paul Hepperly (PR) Mr. Clemens DeKok (IA) Dr. Dan Lubich (NE)
Transgenic Acreage:	Puerto Rico - .15 acres Iowa - .02 acres Nebraska - .02 acres
Dates of Release:	6/19/95 (Puerto Rico) 5/23/95 (Iowa) 7/13/95 (Nebraska)
Isolation method:	Tassels bagged (Puerto Rico) 200 m isolation (Iowa; Nebraska)
Date of Final Termination of Release:	4/08/96 (Puerto Rico)- hand harvested 10/10/95 (Iowa)- hand harvested 10/30/95 (Nebraska)- hand harvested

As requested in the supplemental conditions included with the approval of permit #95-089-04N, I am submitting a summary of the data collected from the field trial involving our Bt tolerant corn, as supervised by Dr. Paul Hepperly, Dr. Dan Lubich and Mr. Clemens deKok, Asgrow Seed Co. The purpose of the trial was to increase seed, advance generations and demonstrate the the agronomic performance of transgenic corn plants.

Means of Containment and Plant Disposition: All sites were machine planted and cleaned out thoroughly after planting the transgenic. After harvest, all seed not used in further breeding experiments was returned or left in the field, and incorporated by plowing or disking to eliminate volunteers. All residue was returned to the plot, incorporated by disking and plowing. Fields in all locations were monitored for volunteer plants. In Puerto Rico the experimental area was rotated out of corn. In Nebraska, the following crop was a Poast tolerant corn crop that was twice treated with Poast herbicide, which would eliminate all the previous years corn, which was all Poast susceptible. In Iowa, the land was rotated to Roundup Ready soybeans, which were hand weeded as well as sprayed with Roundup to eliminate weeds and volunteer corn plants.

Disease and Insect Susceptibility: No changes is morphology, disease or insect resistance was noticed between the transgenic and non-transgenic corn, with the exception of less target insect feeding on the transgenic corn.

Plant Growth Characteristics and Weediness: No differences in seed, seedling or plant growth were noticed that would make the transgenic more likely to be a potential weed problem. In Puerto Rico, some transgenic genotypes exhibited leaf striping of up to 15 %.

Results: Corn lines in Puerto Rico and Iowa were advanced a generation by selfing and cross pollinating and multiplied to provide seed for future breeding experiments. The trials in Nebraska and Iowa were efficacy trials, used to evaluate resistance to various insect species.

I Field Results 1995 - USDA Reports

A. 1995 Summer Testing

Primary Objective

The primary objective was to evaluate the efficacy of several PGS events carrying the Bt gene for European Corn Borer resistance. Secondly, to incorporate this gene into proprietary material of NC+ Hybrids for future development.

Site Selection

NC+ Hybrids
RR #2, Box 190
Hastings, NE 68901
Clay County

Field #4 was selected as the testing site. This field had soybeans as a previous crop which reduced the chances of any volunteer corn. In addition the field was isolated by more than 660 feet in all directions fulfilling USDA gene containment requirements.

Description of Experiment

Overall test dimension was 32 rows x 80' (four 20' ranges). 8 Bt events and 2 control events were evaluated in plots 20' long and the row number varying with seed supply. 12 NC+ proprietary lines were included in 4 row sets. The test was surrounded by guard rows. Overall size was .15 acres.

Chronicle of Events

5-18-95	- Received 1430 seeds from PGS. USDA-APHIS Importation permit #95-097-02n.
6-1,5-95	- Field was disced and then field cultivated with an application of Bullet herbicide.
6-16-95	- Test was planted. USDA-APHIS Release permit #95-097-01N.
6-27,28 -95	- Extremely dry, tank watered the trial.
7-11-95	- Test was field cultivated.
7-12-95	- PGS material dabbed with 1% solution of Liberty herbicide.
7-13-95	- Field was "Hilled" for irrigating.
7-17-95	- Thinned out the Liberty susceptible plants.
7-18-95	- Dabbed a 1% solution of Liberty herbicide on surviving plants as a check.
7-23 to 8-3-95	- Applied 1st generation European corn borer larvae. Approximately 100-125 live larvae per plant.
7-25-95	- Took final counts on survivors of the Liberty herbicide treatment.

8-10 to 8-29-95 - Transferred the Bt even into proprietary material using controlled pollinations.

8-11-95 - Plants evaluated for 1st Brood European Corn Borer damage.

8-16 to 8-29-95 - Inoculated with 2nd Brood European Corn Borer. Approximately 250 live larvae were applied per plant.

9-18 to 9-20-95 - Hand harvested pollinations.

9-21-95 - Killing frost. Most of the pollinations were affected.

10-30-95 - Evaluated 7 plants from 3 events for 2nd Brood European Corn Borer damage. These 3 events were judged best by PGS in their efficacy studies. Four plants and their crosses were kept from two events to be further used in the winter nursery.

11-14-95 - Machine harvested the rest of the test. Seed was composted with husks and cobs from the conditioning plant. The stubble was left standing.

1-29 to 2-9-96 - Evaluated remaining 5 events for 2nd Brood European Corn Borer damage and sent the results to PGS.

2-12-96 - Kept seed from one event and its crosses. All remaining seed was composted with the machine harvested seed.

3-96 - Stubble was chopped and the field disced.

6-1-96 - Field was disced a second time and field cultivated for a soybean crop. Volunteer corn was very sparse (less than 1 plant/100 sq. ft.). Soybean crop was post sprayed and no further volunteer corn was detected.

Results and Observations

- Results on the Liberty application were as predicted with 50% of the plants being tolerant.
- Results on 1st Brood European Corn Borer were quite good with all events showing little or no damage.
- Results on 2nd Brood European Corn Borer were varied with 6 of the 8 events exhibiting some damage. Two other events were quite good and one was selected to continue in our proprietary material.
- All of the Liberty susceptible plants were removed so no difference between transgenic and non-transgenic plants could be observed. However, no unusual physiological traits were observed.
- No unusual pest populations were observed during or following the trial.

HOLDEN'S FOUNDATION SEEDS, INC.
P.O. Box 839, Williamsburg, Iowa 52361
FAX 319 668-2453
319 668-1100

2 May 1997

Field Trial Report
USDA Notification Number: 95-101-12N
(applicant reference: 95 PGS BT2)

Submitted by: Dr. Lori Marshall
Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, Iowa 52361
phone: 319/668-1100
fax: 319/668-2463

Test site Information:
Iowa: One planting was grown from June 1995 to October 1995, in Iowa County, near Holden's Foundation Seeds, Inc. headquarters in Williamsburg, IA. Standard fertilizers and herbicides were used as is typical for our traditional corn breeding research.

Hawaii: Two plantings were grown from September 1995 to April 1996, in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai. Standard fertilizers, herbicides, and irrigation were used as is typical for our traditional corn breeding research.

Summary of experimental results:
Segregation. The plants segregated as expected for a single nuclear gene when treated with glufosinate. In Hawaii, no insects were used to evaluate insect resistance, and standard insecticides were used to control insect pests, so there was no information obtained on the transgenic plants' response to insects. In Iowa, some transgenic plants were artifically inoculated with Eurpoean Corn Borer larvae and showed the expected cosegregation of insect protection and glufosinate tolerance. The transgenic plants are in a backcrossing program in which the Bt genes

are being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and both the selectable marker gene for glufosinate tolerance and the Bt gene for European Corn Borer proection appeared to segregate and express as expected in this wide sampling of genetic backgrounds.

Observations on phenotype. The plants appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparision of transgenic vs non-transgenic corn lines:

Response to generally excellent growing conditions in Iowa - no differences detected; response to standard irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response in Hawaii to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Flowering - no differences detected (Iowa and Hawaii).

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality (Iowa and Hawaii).

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected (Iowa and Hawaii).

The transgenic plants displayed the expected tolerance to glufosinate (Iowa and Hawaii) and protection against European Corn Borer (Iowa) but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

This transgenic material was planted once in Iowa and 2 times in Hawaii

during the period from May 1995 to May 1995. For all plantings, isolation from other corn was used to contain the trial, and no compromise to this containment was observed.

Iowa: June 7, 1995 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.4 acre.

Hawaii: September 12 to October 2, 1995 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.9 acre.

Hawaii: Dec. 29, 1995 to January 15, 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 1.0 acre.

Termination of exerlment

Iowa: June 7, 1995 planting: Hand-pollinated ears were harvested from the trial during the month of October 1995, and held in storage at our Williamsburg, Iowa station. Remaining plant material was disked into the soil in November 1995.

Hawaii: September 12 to October 2, 1995 planting: Hand-pollinated ears were harvested from the trial Dec. 17,1995 to Jan. 8, 1996, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early January 1996. After this initial disking into the soil, the site was left fallow for several months and went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Hawaii: Dec. 29, 1995 to January 15, 1996 planting: Hand-pollinated ears were harvested from the trial April 15 to April 30,1996, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early May 1996. After this initial disking into the soil, the site was left fallow for several months and went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Monitoring for Volunteers

In Iowa, the field work done in the fall of 1995 and spring of 1996 was accomplished in a timely manner to minimize the possibility that volunteers would persist into the 1996 growing season. The field site was carefully monitored and any volunteers that might have survived would have been mechanically removed during extensive roguing throughout the 1996 season.

In Hawaii, during the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings in Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. During the third cycle, no volunteers were observed, as is typical in our standard plantings.

FINAL REPORT

USDA # 95-107-12N (GEI-95-4 IM) IMPORT Permit
USDA # 95-107-11N (GEI-95-5RE) RELEASE Permit
USDA # 95-107-13N (GEI-95-1 IM) IMPORT Permit
USDA # 95-107-10N (GEI-95-2RE) RELEASE Permit
USDA # 95-107-03N (GEI 95-3-MO) INTERSTATE Move

Permit Numbers

USDA:	95-107-12N	(GEI-95-4 IM)	Phenotype: Male Sterile;
USDA:	95-107-11N	(GEI-95-5 RE)	Tolerant to glufosinate ammonium
USDA:	95-107-13N	(GEI-95-1 IM)	Phenotype: Resistance to
USDA	95-107-10N	(GEI-95-2RE)	Lepidopteran insect pests.
USDA	95-107-03N	(GEI 95-3-MO)	

EXPERIMENT DESCRIPTION AND RESULTS

The objectives of the plantings were:

1. Verify the genotypes and take notes on plant characteristics including male sterility.

2. Test various genotypes for resistance to Lepidoptera artificial infestation.

3. Increase seed by hand pollination.

Plots were located in a farm isolation near Slater, Story County, Iowa. trial was planted May 30,1995.

Area covered by transgenic material under permits (see below)

USDA # 95-107-12N (GEI-95-4 IM) IMPORT Permit
USDA # 95-107-11N (GEI-95-5RE) RELEASE Permit
USDA # 95-107-13N (GEI-95-1 IM) IMPORT Permit
USDA # 95-107-10N (GEI-95-2RE) RELEASE Permit
USDA # 95-107-03N (GEI 95-3-MO) INTERSTATE Move

was 2 acres and included by construct:
SEE ATTACHED PAGES.

Harvest was September 30, and the plots were destroyed October 15, All notes and data were taken successfully in all materials.

General Field Observations

Plot observations indicated no differences in disease damage between transgenic and related non-transgenic plants. Resistance to Lepidopt was shown by transgenic material carrying the specific constructs fo: trait. Male sterility was shown by transgenic material carrying the specific constructs for this trait.

No characteristics that may lead to increased weediness were observed..

CONFIDENTIAL BUSINESS INFORMATION DELETED

Disposal

Remaining plant material after harvest was disked into soil.

Volunteers

No voulnteer plants were observed in the fall of 1995 or spring of 199 These observations are consistent with those of nontransgenic corn in environment.

NORTHRUP KING COMPANY
FIELD TEST REPORT FOR TRANSGENIC CROPS

Permit \ Notification Number: 95-261-07n (CBH-351)

SITE INFORMATION: *Indicate which site this report applies.*

Name: Northrup King Co.
Address: 7050 Kaumualii Highway
City, State, Zip: Kekaha, HI 96752

County: Kauai Acreage of transgenics: .028 Acres
Planting Date 10/19/95 Isolation: Spatial
Harvest Date: 2/2/96 Disposal: Seed harvest, plowing, herbicides next seaso

OBSERVATIONS: *Compare the control verses the transgenic lines for obvious differences in the following areas. Note percentage of plants affected. If no differe are detected, please indicate so in each criteria.*

- Disease Susceptibility (other than diseases not specifically engineered for resistance):

None

- Insect Susceptibility (other than insects not specifically engineered for resistance):

None

- Weediness Characteristics (e.g., volunteers, tasseling, seed production, etc.):

None

- Stand or Germination:

None

► Plant Phenotype:

Standard corn, indistinguishable from non transgenic corn, except for the transgen effect.

► Effects on Normal Soil Organisms:

None

► Effects on Beneficial Insects:

No negative effects

► Other Observations:

None noted

Please send completed
Field Test Reports to:

Northrup King Company
Government Regulations
P.O. Box 959
Minneapolis, MN 55440

NORTHRUP KING COMPANY
FIELD TEST REPORT FOR TRANSGENIC CROPS

Permit \ Notification Number: 95-261-07n (CBH-351)

SITE INFORMATION: *Indicate which site this report applies.*

Name: Northrup King Co.
Address: 7050 Kaumualii Highway
City, State, Zip: Kekaha, HI 96752

County: Kauai Acreage of transgenics: .0377 Acres
Planting Date 2/9/96 Isolation: Spatial
Harvest Date: 6/3/96 Disposal: Seed harvest, plowing, herbicides next seasc

OBSERVATIONS: *Compare the control verses the transgenic lines for obvious differences in the following areas. Note percentage of plants affected. If no differ are detected, please indicate so in each criteria.*

- Disease Susceptibility (other than diseases not specifically engineered for resistance):

None

- Insect Susceptibility (other than insects not specifically engineered for resistance):

None

- Weediness Characteristics (e.g., volunteers, tasseling, seed production, etc.

None

- Stand or Germination:

None

- Plant Phenotype:

Standard corn, indistinguishable from non transgenic corn, except for the transgen effect.

- Effects on Normal Soil Organisms:

None

- Effects on Beneficial Insects:

No negative effects

- Other Observations:

None noted

Please send completed
Field Test Reports to:

Northrup King Company
Government Regulations
P.O. Box 959
Minneapolis, MN 55440

FINAL REPORT

USDA# 95-272-08N
USDA# 95-272-05N

Gregory B. Parker
Golden Harvest Seeds, Inc.

Permit Numbers

USDA: 95-272-08N phenotype: Lepidopteran insect resistance
Golden Harvest: GH-95-21re

USDA: 95-272-05N phenotype: Male Sterile, Tolerant to glufosinate ammonium
Golden Harvest: GH-95-11re

Experiment Description and Results

The objective of the planting was to produce seed for testing and to introgress the genes of interest into proprietary germplasm.

Plots were located on Molokai, Hawaii (Maui County). The trial was planted 11/28/95.

Transgenic area covered by lines planted under 95-272-08N was 0.021 acres, and included, by construct:

pRVA9906:
[ ] CBI DELETED

pRVA9909 + [ ] CBI DELETED
[ ] CBI DELETED

Transgenic area covered by lines planted under 95-272-05N was 0.017 acres, and included only line MS-3, construct pVE108.

Harvest was 2/27/96, and plots were destroyed 3/14/96. Generation advancement was successful for all lines.

General Field Observations

Nursery observations indicated no differences in disease or insect damage between transgenic plants and related non-transgenic lines. No characteristics that may lead to increased weediness were observed.

Disposal

Remaining plant material after harvest was disked into soil.

Volunteers

Three observations were made. On 3/25, 15 plants/m^2 were observed, and disked 1 day later. On 4/6, 12 plants/m^2 were observed, and disked 1 day later. On 4/22, 2 plants/m^2 were observed, and disked 4 days later. On the last observation, 5/10, no volunteers were observed. These observations are consistent with those of non-transgenic corn in this environment.

FINAL REPORT
USDA# 95-272-08N

Gregory B. Parker
Golden Harvest Seeds, Inc.

Permit Numbers

USDA: 95-272-08N phenotype: Lepidopteran insect resistance
Golden Harvest: GH-95-21re

Experiment Description and Results

The objective of the planting was to produce seed for testing and to introgress the genes of interest into proprietary germplasm.

Plots were located on Molokai, Hawaii (Maui County). The trial was planted 3/1/96.

Transgenic area covered by lines planted under 95-272-08N was 0.003 acres, total acreage was 0.009, and included:

pRVA9909 + [ ] CBI DELETED
[ ] CBI DELETED

Harvest was 6/13/96, and plots were destroyed 6/20/96. Mice invaded the plots shortly before harvest resulting in loss of most pollinations. However enough seed was obtained to initiate the next generation.

General Field Observations

Nursery observations indicated no differences in disease or insect damage between transgenic plants and related non-transgenic lines. No characteristics that may lead to increased weediness were observed.

Disposal

Remaining plant material after harvest and initial plot destruction was disked into soil 7/9/96.

Volunteers

Monitoring for volunteers is in progress. Any differences between transgenic and non-transgenic plots will be reported.

B. 1995/96 Winter Nursery

Primary Objective

The primary objective of the winter testing program is to continue incorporating the PGS Bt event into additional NC+ proprietary lines. In addition, backcrossing generations will be initiated and continued with lines converted in the summer.

Site Selection

Hawaiian Research (sub-contractor)
Attn: Peter Eickhorn or Barry O'Keefe
P.O. Box 40
Mauna Loa Hwy. West
Kuanakakai Molokai, Hawaii
Maui County
Telephone: 808-553-5070
Fax: 808-553-5436
Hawaiian research selected the field to put this nursery which fulfilled the USDA gene containment isolation requirements.

Description of Experiment

This nursery contains 102 - 20' x 30" rows. 80 rows are designed for backcrossing and organized in 5 row sets with the 1st row the finished line followed by four rows of the developing Bt material. 8 rows are selves from the PGS event being used for incorporating the Bt gene. 14 rows are new lines to transfer the Bt gene into. Overall size was .12 acres.

Chronicle of Events

10-6-95 - Interstate Movement and Release Notification letter sent to USDA-APHIS.
11-13-95 - Acknowledgment by USDA-APHIS on Interstate Movement and Release (#95-284-13N).
11-14-95 - Approximately 4000 seeds of Bt conversions were sent to Hawaiian Research.
11-20-95 - Hawaiian research planted the nursery in Field #13C-2.
12-18 to 12-22-95 - Estimate Bt material was treated with a 1% Liberty herbicide solution to identify and thin out non Bt plants.
1-15 to 1-28-96 - Initial backcross made by controlled pollinations and new lines incorporated with the Bt event.
3-9-96 - Lines were hand harvested and ears dried.
3-10-96 - Remaining plants and seed shredded and disced by Hawaiian research.

3-12-96 - Ears were shelled and seed packaged for a spring planting by Hawaiian Research. (Release permit #95-284-13N).
Remaining seed (approximately 8000 seeds) were sent to NC+ Hybrids for efficacy studies. (Interstate Movement Permit #95-284-13N)

3-15-96 - Hawaiian Research reports volunteer corn at 15 plants/square meter.
- Spring nursery planted by Hawaiian Research. (See C. 1996 Spring Nursery)

3-26-96 - Field was disced.

4-4-96 - Volunteer corn reported at 12 plants/square meter.

4-5-96 - Field disced.

4-22-96 - Volunteer corn reported at 2 plants/square meter.

4-26-96 - Field disced.

5-10-96 - No additional volunteer plants observed by Hawaiian Research.

Results and Observations

- Hawaiian Research reported no unusual events or insect/plant pests in this nursery. Individual staff members from NC+ observed no unusual plant characteristics or oddities while pollinating this nursery.

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 7 May 1996

Site of Release: ICI Seeds Research Farm, 2369 330th Street, Slater, IA 50244

Maximum Acreage: 0.164

Site Contact: Fen Huang, Trait Breeder

Purpose of Release: Introgress Bt gene into elite maize germplasm

Results: All planned pollinations were accomplished.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst breeders and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by covering the tassels and ear shoots until their use for controlled pollinations by the breeder. After final use, tassels were removed from the plant.

Date of Release Termination: 18 November 1996

Means of Plant Disposition: Selected ears were hand-harvested, shelled in the lab, and cobs were returned to plot area. Shelled seed was put into storage for use in breeding programs. All other remaining plant material was composted in the plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 30 May 1996

Site of Release: ICI Seeds Research Farm, R.R. 4, Box 46, Sleepy Eye, MN 56085

Maximum Acreage: 0.004

Site Contact: Chris Reitan, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 13 September 1996

Means of Plant Disposition: Incineration of all plant material at designated site on the farm.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

Garst ref: Y:\APHIS\PERMITS\9532002N\RPTMN.DOC

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 30 May 1996

Site of Release: ICI Seeds Research Farm, 2560 Nora Road, Cottage Grove, WI 53527

Maximum Acreage: 0.004

Site Contact: Jeff Pomeroy, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 21 October 1996

Means of Plant Disposition: Composting of all plant material within the plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 30 May 1996

Site of Release: ICI Seeds Research Farm, 2369 330th Street, Slater, IA 50244

Maximum Acreage: 0.053

Site Contact: Von Kaster, Entomologist

Purpose of Release: Evaluate efficacy of Bt gene against European corn borer and corn earworm

Results: Bt transgenic plants were manually-infested with European corn borer at mid-whorl stage of plant development. Visual ratings for leaf feeding resistance were taken approximately 21 days post-infestation. All plants were manually-infested at pollen shed with European corn borer and the degree of tunneling was determined approximately 45 days post-infestation. Little to no damage from either European corn borer infestation was observed in the transgenic plots, while the non-Bt checks showed severe damage. At silking, Bt transgenic plants were manually infested with neonate corn earworm larvae. The transgenic plots exhibited reduced silk feeding and tip damage when compared to the non-Bt checks.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB) and corn earworm (CEW). Plants engineered with the Bt gene showed little or no damage by ECB and reduced damage by CEW. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by complete removal of all tassels prior to anthesis.

Date of Release Termination: 19 November 1996

Means of Plant Disposition: Composting of all plant material within the field plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 30 May 1996

Site of Release: ICI Seeds Research Farm, 2369 330th Street, Slater, IA 50244

Maximum Acreage: 0.025

Site Contact: Von Kaster, Entomologist

Purpose of Release: Evaluate efficacy of Bt gene against black cutworm

Results: Bt transgenic plants were manually-infested with 4th instar black cutworm larvae at the V3 stage of plant development. Percentage feeding and percentage cut plants were recorded. There were no significant differences in either % feeding or % cutting between transgenic and non-transgenic plants.

Observations:

Insect susceptibility: The duration of this trial was not of sufficient length to observe differences between the transgenic and non-transgenic plants, except for the test insect, black cutworm.

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: Although of short duration, in this trial there were no observable differences between the transgenic and non-transgenic lines with respect to emergence and early season vigor.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by destruction of the trial before anthesis.

Date of Release Termination: 8 July 1996

Means of Plant Disposition: Composting of all plant material within the field plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

Garst ref: Y:\APHIS\PERMITS\9532002N\RPTIA3.DOC

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 31 May 1996

Site of Release: ICI Seeds Research Farm, 16275 US Highway 34, P.O. Box 191A, Galva, IL 61434

Maximum Acreage: 0.004

Site Contact: Brian Alliger, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 25 October 1996

Means of Plant Disposition: Composting of all plant material at designated site on the farm.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 3 June 1996

Site of Release: ICI Seeds Research Farm, 10476 West Husker Highway, Wood River, NE 68883

Maximum Acreage: 0.03

Site Contact: Chandler Mazour, Development Agronomist

Purpose of Release: Evaluate efficacy of Bt gene against European corn borer.

Results: Bt transgenic plants were manually-infested with European corn borer at mid-whorl stage of plant development. Visual ratings for leaf feeding resistance were taken approximately 21 days post-infestation. All plants were manually-infested at pollen shed with European corn borer and the degree of tunneling was determined approximately 45 days post-infestation. Little to no damage from either European corn borer infestation was observed in the transgenic plots, while the non-Bt checks showed severe damage.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by complete removal of all tassels prior to anthesis.

Date of Release Termination: 21 October 1996

Means of Plant Disposition: Composting of all plant material at designated site on the farm.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 3 June 1996

Site of Release: ICI Seeds Research Farm, 10476 West Husker Highway, Wood River, NE 68883

Maximum Acreage: 0.004

Site Contact: Chandler Mazour, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 9 October 1996

Means of Plant Disposition: Composting of all plant material at designated site on the farm.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 4 June 1996

Site of Release: ICI Seeds Research Farm, R. R. 2, Box 16, Marshall, MO 65340

Maximum Acreage: 0.004

Site Contact: Jim Deutsch, Station Manager, and Chris Hummel, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 22 October 1996

Means of Plant Disposition: Grain and cobs were hand-harvested and burned. All remaining plant material was left in plot area to compost.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

Garst ref: Y:\APHIS\PERMITS\9532002N\RPTMO1.DOC

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 4 June 1996

Site of Release: ICI Seeds Research Farm, R. R. 2, Box 16, Marshall, MO 65340

Maximum Acreage: 0.03

Site Contact: Jim Deutsch, Station Manager, and Chris Hummel, Development Agronomist

Purpose of Release: Evaluate efficacy of Bt gene against southwestern corn borer and fall armyworm.

Results: Bt transgenic plants were manually-infested with either southwestern corn borer or fall armyworm at mid-whorl stage of plant development. Visual ratings for leaf feeding resistance were taken approximately 21 days post-infestation. All plants were manually-infested at pollen shed with southwestern corn borer and the degree of tunneling was determined approximately 45 days post-infestation. Little to no damage from either southwestern corn borer infestation was observed in the transgenic plots, while the non-Bt checks showed severe damage. Damage from the fall armyworm infestation of the non-Bt checks was minimal, so no efficacy data were taken.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by southwestern corn borer. Plants engineered with the Bt gene showed little or no damage by southwestern corn borer. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by complete removal of all tassels prior to anthesis.

Date of Release Termination: 22 October 1996

Means of Plant Disposition: Grain and cobs were hand-harvested and burned. All remaining plant material was left in plot area to compost.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

APHIS Environmental Release Report

Permit Number: 95 - 320 - 02N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 14 June 1996

Site of Release: ICI Seeds Research Center, 2369 330th Street, Slater, IA 50244

Maximum Acreage: 0.012

Site Contact: Jeff Sernett, Development Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Herbicide tolerance: Pre- and post-emergent herbicide was applied. No damage to transgenic and non-transgenic lines was observed by Garst agronomists.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

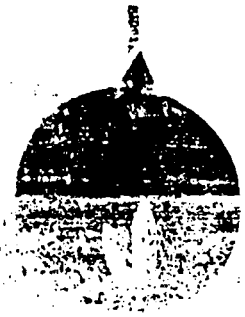
Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 19 November 1996

Means of Plant Disposition: Composting of all plant material within the plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress



Golden Harvest Seeds, Inc.

Gregory B. [illegible]
Plant Technology Manager
Golden Harvest Seeds, Inc.
25922 595th Avenue
Nevada, Iowa 50201
Phone 515-382-4503
Fax 515-382-4801

[illegible]
[illegible]
Animal and Plant Health Inspection Service
Biotechnology, Biologics, and Environmental Protection
[illegible]

Dear Ms. Hammerer:

[illegible]

Please contact [illegible] information is needed. Thank you

Sincerely,

[illegible]

FINAL REPORT

USDA# 96-059-02N

Gregory B. Parker
Golden Harvest Seeds, Inc.
8/15/97

Permit Numbers

USDA: 96-059-02N phenotype: Lepidopteran insect resistance
Golden Harvest: 96-057RM

Experiment Description and Results

May, 1996, Hawaii
The objective of this planting was to produce seed for testing and to introgress the genes of interest into proprietary germplasm. Plots were located on Molokai, Hawaii (Maui County). The trial was planted 5/13/96 over an area of 0.100 acres (transgenic). An isolation distance of 200 meters was maintained from open-pollinated corn not destroyed. Lines planted included CBH304, CBH351, and CBH354. The constructs of these lines are listed in the notification. Harvest occurred over the period 8/7/96 - 8/20/96, and plots were destroyed within 1 month of the last harvest. Generation advancement was successful.

June, 1996, Hawaii
The objective of this planting was to produce seed for testing and to introgress the genes of interest into proprietary germplasm. Plots were located on Molokai, Hawaii (Maui County). The trial was planted 6/27/96 over an area of 0.036 acres (transgenic). An isolation distance of 200 meters was maintained from open-pollinated corn not destroyed. Lines planted included CBH351 and CBH354. The constructs of these lines are listed in the notification. No seed was harvested from this nursery, since it was a backup to the May-planted nursery described above, and that nursery was successfully harvested. Plots were destroyed in October, 1996.

Mainland U.S. Locations, 1996

The objective of these plantings was to assess efficacy of the lines in controlling European Corn Borer. Plots were located in Iowa, Nebraska, Missouri, and Kansas.

Location	Lines	Planting Date	Transgenic Area
Story County, IA	CBH351, CBH304, CBH354	5/6/96	0.012 acres
Ray County, MO	CBH351, CBH304, CBH354	5/19/96	0.010 acres
Douglas County, NE	CBH351, CBH304, CBH354	5/20/96	0.017 acres
Haskell County, KS	CBH351	5/29/96	0.019 acres

At the Nebraska location, an isolation distance of 200 meters was maintained from open-pollinated corn not destroyed. At the other locations, the transgenic corn plants were detasseled prior to pollen shed. At all locations, European Corn Borer were controlled by the transgenic plants. No seed was harvested from any of these locations. Plots were chopped and disked at the conclusion of the experiments.

General Field Observations

In none of the field releases described above were any differences in disease or insect damage between transgenic plants and related non-transgenic lines observed. No characteristics that may lead to increased weediness were observed.

Disposal

Remaining plant material after harvest was disked into soil.

Volunteers

Volunteer plants, where present, occurred in numbers normal for corn in growing in the respective areas. In Hawaii, fields were irrigated following destruction to stimulate germination, and any resultant plants mechanically destroyed by disking. This process was repeated until no volunteers were observed.

NORTHRUP KING COMPANY
FIELD TEST REPORT FOR TRANSGENIC CROPS

Permit \ Notification Number: **#96-075-04N** (PGS 351)

SITE INFORMATION: *Indicate which site this report applies.*

Name: **Northrup King Co.**
Address: **317 330th St.**
City, State, Zip: **Stanton, MN 55018**

County: Goodhue
Planting Date 5/21/96
Harvest Date: _____

Acreage of transgenics: .022A (22 rows)
Isolation: Spatial
Disposal: Seed harvest, plowing, herbicides next season.

Type of trial (efficacy, yield,tc): **Nursery, efficacy**

OBSERVATIONS: *Compare the control verses the transgenic lines for obvious differences in the following areas. Note percentage of plants affected. If no differences are detected, please indicate so in each criteria.*

- Disease Susceptibility (other than diseases not specifically engineered for resistance):

None

- Insect Susceptibility (other than insects not specifically engineered for resistance):

None

- Weediness Characteristics (e.g., volunteers, tasseling, seed production, etc.):

None

- Stand or Germination:

No difference from non transgenics - ~20k/row

- Plant Phenotype:

Standard corn, indistinguishable from non transgenic corn, except for the transgene effect.

- Effects on Normal Soil Organisms:

None

- Effects on Beneficial Insects:

No negative effects

- Other Observations:

Plantings were for increasing and advancing seed or for efficacy screening on Lepidopterans.

Please send completed Field Test Reports to:

Northrup King Company
Government Regulations
P.O. Box 959
Minneapolis, MN 55440

NORTHRUP KING COMPANY
FIELD TEST REPORT FOR TRANSGENIC CROPS

Permit \ Notification Number: #96-075-04N (PGS 351)

SITE INFORMATION: *Indicate which site this report applies.*

Name: Northrup King Co.
Address: 306 Meadow Dr.
City, State, Zip: St. Joseph, IL

County: Champaign Acreage of transgenics: .022A (22 rows)
Planting Date 5/ /96 Isolation: Spatial
Harvest Date: _____ Disposal: Seed harvest, plowing, herbicides next season.

Type of trial (efficacy, yield,tc): Nursery, efficacy

OBSERVATIONS: *Compare the control verses the transgenic lines for obvious differences in the following areas. Note percentage of plants affected. If no differences are detected, please indicate so in each criteria.*

- Disease Susceptibility (other than diseases not specifically engineered for resistance):

None

- Insect Susceptibility (other than insects not specifically engineered for resistance):

None

- Weediness Characteristics (e.g., volunteers, tasseling, seed production, etc.):

None

- Stand or Germination:

No difference from non transgenics - ~20k/row

▸ Plant Phenotype:

Standard corn, indistinguishable from non transgenic corn, except for the transgene effect.

▸ Effects on Normal Soil Organisms:

None

▸ Effects on Beneficial Insects:

No negative effects

▸ Other Observations:

Plantings were for increasing and advancing seed or for efficacy screening on Lepidopterans.

Please send completed
Field Test Reports to:

Northrup King Company
Government Regulations
P.O. Box 959
Minneapolis, MN 55440

NORTHRUP KING COMPANY
FIELD TEST REPORT FOR TRANSGENIC CROPS

Permit \ Notification Number: 96-075-04N (CBH-351)

SITE INFORMATION: *Indicate which site this report applies.*

Name: Northrup King Co.
Address: 7050 Kaumualii Highway
City, State, Zip: Kekaha, HI 96752

County: Kauai Acreage of transgenics: .067 Acres
Planting Date 6/20/96 Isolation: Spatial
Harvest Date: _____ Disposal: Seed harvest, plowing, herbicides next season.

OBSERVATIONS: *Compare the control verses the transgenic lines for obvious differences in the following areas. Note percentage of plants affected. If no differences are detected, please indicate so in each criteria.*

- Disease Susceptibility (other than diseases not specifically engineered for resistance):

None

- Insect Susceptibility (other than insects not specifically engineered for resistance):

None

- Weediness Characteristics (e.g., volunteers, tasseling, seed production, etc.):

None

- Stand or Germination:

None

▸ Plant Phenotype:

Standard corn, indistinguishable from non transgenic corn, except for the transgene effect.

▸ Effects on Normal Soil Organisms:

None

▸ Effects on Beneficial Insects:

No negative effects

▸ Other Observations:

None noted

Please send completed
Field Test Reports to:

Northrup King Company
Government Regulations
P.O. Box 959
Minneapolis, MN 55440

APHIS Environmental Release Report

Permit Number: 96 - 086 - 07N

Permittee: Dr. Paul Bullock, Garst Seed Company, 2369 300th Street, Slater, IA 50244 [515-685-5000]

Date of Release: 8 July 1996

Site of Release: ICI Seeds Research Plots, Highway 141 West, Coon Rapids, IA 50058

Maximum Acreage: 0.004

Site Contact: John Pieper, Sales Agronomist

Purpose of Release: Demonstration of Bt efficacy

Results: Bt transgenic plants were manually-infested with European corn borer and little to no damage was observed.

Observations:

Insect susceptibility: As expected, non-transgenic plants were susceptible to feeding by European corn borer (ECB). Plants engineered with the Bt gene showed little or no damage by ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetle, and grasshoppers. In addition, casual observations by experienced Garst agronomists and entomologists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (e.g. honeybees).

Disease susceptibility: No pesticides were applied to control plant diseases. Garst agronomists observed no differences between transgenic and non-transgenic lines in their susceptibility to disease.

Herbicide tolerance: Pre- and post-emergent herbicide was applied. No damage to transgenic and non-transgenic lines was observed by Garst agronomists.

Physical characteristics: There were no observable differences between the transgenic and non-transgenic lines with respect to the following characteristics that are observed routinely in the course of traditional corn field tests and breeding programs:

Flowering dates, plant height, plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle/width.

Means and Effectiveness of Containment Measures: The experiment was grown in a clearly defined area and containment accomplished by a temporal shift where the flowering period of the transgenic plants did not coincide with the presence of receptive silks on plants within 200 meters.

Date of Release Termination: 17 October 1996

Means of Plant Disposition: Composting of all plant material within the plot area.

Time and Method of Monitoring for Volunteer Plants: Pending, still in progress

Number of Volunteers Observed and Action Taken: Pending, still in progress

HOLDEN'S FOUNDATION SEEDS, INC.
P.O. Box 839, Williamsburg, Iowa 52361
FAX 319 668-2453
319 668-1100

2 May 1997

Field Trial Report
USDA Notification Number: 96-103-03N
(applicant reference: 96R PGS BT)

Submitted by: Dr. Lori Marshall
Holden's Foundation Seeds, Inc.
201 N. Maplewood, P.O. Box 839
Williamsburg, Iowa 52361
phone: 319/668-1100
fax: 319/668-2453

NOTE: THIS IS A PARTIAL REPORT, ONE SITE IS STILL IN PROGRESS, AND THE POST-HARVEST MONITORING FOR SOME HAWAII SITES IS NOT YET COMPLETE.

Test site information:

Iowa: One planting was grown from June 1996 to October 1996, in Iowa County, near Holden's Foundation Seeds, Inc. headquarters in Williamsburg, IA. Standard fertilizers and herbicides were used as is typical for our traditional corn breeding research.

Hawaii: Multiple plantings were grown from May 1996 to May 1997, in Maui County, Molokai, Hawaii, near Holden's Foundation Seeds, Inc. office in Kaunakakai. Standard fertilizers, herbicides, and irrigation were used as is typical for our traditional corn breeding research.

Illinois: One planting was grown from May 1996 to October 1996, in Henry County, in a rural area near Geneseo, IL. Standard fertilizers and herbicides were used as is typical for traditional corn breeding research.

Indiana: One planting was grown from May 1996 to October 1996, in Johnson County, near Holden's Foundation Seeds, Inc. office in Whiteland,

IN. Standard fertilizers and herbicides were used as is typical for traditional corn breeding research.

(Sites listed in the original notification for Carroll and Hancock Counties in Indiana were cancelled and not used.)

Nebraska: One planting was grown from May 1996 to October 1996, in Polk County, near Holden's Foundation Seeds, Inc. office in Stromsburg, NE. Standard fertilizers and herbicides were used as is typical for traditional corn breeding research.

Pennsylvania: One planting was grown from May 1996 to October 1996, in Lycoming County, near Jersey Shore, PA. Standard fertilizers and herbicides were used as is typical for traditional corn breeding research.

Summary of experimental results:

Segregation: The plants generally segregated as expected for a single nuclear gene when treated with glufosinate. In Hawaii, no insects were used to evaluate insect resistance, and standard insecticides were used to control insect pests, so there was no information obtained on the transgenic plants' response to insects. In Iowa and Illinois, some transgenic plants were artifically inoculated with Eurpoean Corn Borer larvae and showed the expected cosegregation of insect protection and glufosinate tolerance. The transgenic plants are in a backcrossing program in which the Bt genes are being crossed into a set of Holden's proprietary inbred lines (designated "LH" followed by a number). This set includes inbreds which represent very diverse germplasm types, and both the selectable marker gene for glufosinate tolerance and the Bt gene for European Corn Borer proection appeared to segregate and express as expected in this wide sampling of genetic backgrounds.

Observations on phenotype. The plants generally appeared completely normal in their phenotype. The rows containing transgenic plants were planted adjacent to rows of nontransgenic standard corn inbred lines. During the course of the growing season the following circumstances allowed comparision of transgenic vs non-transgenic corn lines:

Response to planting in a cold, wet spring followed by good growing conditions in Iowa - no differences detected; response to standard

Irrigation and Hawaii's warm soil temperatures during germination period - no differences detected.

Response in Hawaii to standard "fertigation" (fertilizer applied through irrigation system) - no differences detected.

Flowering - no differences detected (Iowa and Hawaii).

Seed set on hand-pollinated ears - no differences detected. Transgenic plants had good seed set and kernel quality (Iowa and Hawaii). Ear worm damage was noted on transgenic ears, indicating that the Bt genes as expressed did not provide dramatic protection against ear worms.

Late-season plant appearance (susceptibility to stalk-rotting diseases, ear molds and smuts, and general plant vigor) - no differences detected (Iowa, Indiana, Nebraska, Illinois, Pennsylvania and Hawaii).

The transgenic plants displayed the expected tolerance to glufosinate (Iowa and Hawaii) and excellent protection against European Corn Borer (Iowa, Indiana, Nebraska, Illinois, and Pennsylvania) but no other apparent change in phenotype (including no change in disease status).

There were no unanticipated differences in morphology, weediness, flowering (silk) characteristics, or long-term survivability of progeny observed.

Size and containment for trial

Iowa site: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 1.5 acre. Containment= isolation.

Hawaii: May/June 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.6 acre. Containment= isolation.

Hawaii: August/September 1996 planting: Tranegenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 1.3 acre. Containment= isolation.

Hawaii: November 24, 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.2 acre. Containment= isolation.

Hawaii: January/February 1997 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied a total of approximately 6 acres, spread over several fields in the Kaunakakai area. Containment= isolation.

Illinois site: May/June 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.2 acre. Containment= isolation.

Indiana: May/June 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.2 acre. Containment= detasseling.

Nebraska: May/June 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.2 acre. Containment= detasseling.

Pennsylvania: May/June 1996 planting: Transgenic plants and non-transgenic plants to which the transgenic plants were crossed occupied approximately 0.2 acre. Containment= detasseling.

Termination of exeriment

Iowa, Illinois, Indiana, Nebraska, Pennsylvania sites - May/June 1996 plantings: Hand-pollinated ears were harvested from the Iowa site during the month of October 1995, and held in storage at our Williamsburg, Iowa station. No ears were collected from the other sites. Remaining plant material was disked into the soil at all sites in the fall of1996.

Hawaii: May/June 1996 planting: Hand-pollinated ears were harvested from the trial in August and September, 1996, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early September 1996. After this initial disking into the soil, the site was left fallow for several months and went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Hawaii: August/September 1996 planting: Hand-pollinated ears were harvested from the trial in November and December, 1996, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in early January 1997. After this initial disking into the soil, the site was left fallow for several months and went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Hawaii: November 24, 1996 planting: Hand-pollinated ears were harvested from the trial March 4,1997, and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears was disked into the soil in mid-March 1997. After this initial disking into the soil, the site was left fallow for several months and went through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Hawaii: January/February 1997 planting: Hand-pollinated ears were harvested from the trial starting in April1997 (and harvest is still continuing in May 1997), and held in storage at our Kaunakakai, Molokai station. Remaining plant material and seed from open-pollinated ears will disked into the soil in mid-May 1997. After this initial disking into the soil, the site will be left fallow for several months and will go through 3 cycles of (1) irrigation, (2) fallow for about 3 weeks, (3) disking to destroy any seedlings.

Monitoring for Volunteers

At the Iowa, Illinois, Indiana, Nebraska, and Pennsylvania sites, field work done in the fall of 1996 and yet-to-be completed in the spring of 1996 will minimize the possibility that volunteers will persist into the 1997 growing season. The field sites will be carefully monitored and any volunteers that might have survived will be mechanically removed.

In the multiple Hawaii plantings, during the first irrigation-fallow-disking cycle, many volunteers were observed, as is typical for our standard corn plantings in Hawaii. During the second irrigation-fallow-disking cycle, a handful of volunteers were observed, as is typical in our standard plantings. During the third cycle, no volunteers were observed, as is typical in our standard plantings. These 3 cycles have not yet been completed for the January/February 1997 plantings.

FINAL REPORT

USDA # 96 -107-03N (GEI -96-01) IMPORT Permit
USDA # 96 -107-04N (GEI -96-02) RELEASE Permit

Permit Numbers

USDA # 96 -107-03N (GEI -96-01) Phenotype:Resistance
USDA # 96 -107-04N (GEI -96-02) to Lepidoptera
insect pests

EXPERIMENT DESCRIPTION AND RESULTS

The objectives of the plantings were:

1. Test the efficacy of various transgenic constructs for resistance to Lepidoptera attack.

2. Test normal hybrids and their near isogenic transgenic counterparts for yield, resistance to Lepidoptera infestation and other agronomic traits. Treatments included plots artificially infested and non infested with Lepidotera and insecticide applications.

3. NonTarget Insect evaluations on normal and transgenic genotypic counterparts.

4. In-Field Corn characterization of corn plants expressing Cry9C and PAT proteins in corn tissues during a field season.

5. Genotype characterization of corn plants expressing Cry9C and PAT proteins in corn plants of different genotypes during the growing season.

Plots were located in an isolation near Johnston, Polk County, Iowa.
The trial was planted May 30,1996.
Area covered by transgenic material under permits (see below)
USDA # 96 -107-03N (GEI -96-01) IMPORT Permit
USDA # 96 -107-04N (GEI -96-02) RELEASE Permit

was 2 acres and included by construct:
SEE ATTACHED PAGES.

Harvest was October 31, 1996.

Plots were destroyed October 31,1996.

All notes and data were taken successfully in all materials.

General Field Observations:

Plot observations indicated no differences in disease damage between transgenic and related non-transgenic plants. Resistance to Lepidoptera was shown by transgenic material carrying the specific constructs for this trait. No characteristics that may lead to increased weediness were observed.

CONFIDENTIAL BUSINESS INFORMATION DELETED

Disposal
Remaining plant material after harvest was disked into soil.

Volunteers
Observations will be made in the spring of 1997

WYFFELS HYBRIDS

RESEARCH AND DEVELOPMENT CENTER
13344 U.S Highway 6
Geneseo, IL 61254-8487
(309) 944-8334
FAX (309) 944-8337

THIS DOCUMENT CONTAINS CONFIDENTIAL BUSINESS INFORMATION

August 14, 1997

Animal and Plant Health Inspection Services
Biotechnology, Biologics, and Environmental Protection
Biotechnology Permits
4700 River Road, Unit 147
Riverdale, MD 20737-1237

RE: FINAL REPORT
USDA Reference Number: 96-115-05N

Responsible Researcher:

Wyffels Hybrids, Inc.
13344 U.S. Hwy 6
Geneseo, IL 61254

James S. Parks
Phone: 309-944-8334
Fax: 309-944-8337

Permit Number:

USDA# 96-115-05N phenotype: Resistance to Lepidopteran insect pests;
Tolerance to glufosinate ammonium herbicide

Experiment Description and Results:

The objective of the planting was to evaluate gene efficacy and to introgress the gene of interest into proprietary germplasm.

Plots were located at Geneseo, Illinois (Henry County). The material was planted June 14, 1996.

Transgenic area covered by lines planted under 96-115-05N was 0.02 acres, and included, by construct:

pRVA9906	
[CBH-304]	CBI
pRVA9909 + [pDE 110]	CBI
[CBH-351; CBH-354]	CBI

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CONFIDENTIAL BUSINESS INFORMATION

Experiment Description and Results: (continued)

This transgenic area was isolated from other corn by a distance of over 900 feet.

Harvest was conducted September 30, 1996 and plots were destroyed November 1, 1997. Generation advancement was successful for all lines.

General Field Observations

Trial and nursery observations indicated **no differences** in germination, seedling vigor, overall phenotype, disease or insect damage between transgenic plants and related non-transgenic lines. No characteristics that may lead to increased weediness were observed.

Disposal

Remaining plant material after harvest was disked into the soil.

Volunteers

Three observations were made for volunteer plants in the following growing season crop of non-transgenic soybeans. On May 22, 1997, approximately 1 plant/m^2 was observed and removed 1 day later by hoeing. The second observation was made on June 5, 1997 and a few scattered volunteer plants were observed and promptly removed by hoeing. The third observation was made on June 27, 1997 and no volunteer plants were observed. These observations are consistent with those of non-transgenic corn in this environment.

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 95-094-03N

Project: CBH202; CBH203; CBH301; CBH306, CBH304; CBH305; CBH351, CBH353

Planting Site: Aurora, Kane County, IL

Responsible Party:
Marty McGregor
Cargill Hybrid Seeds
2600 W. Galena Blvd.
Aurora, IL 60506
630-801-2325
630-801-2345 (FAX)

Planting Date: May 17, 1995

Harvest Date: October 10, 1996

Number of Transgenic Rows Planted: 18

Total Acreage: 0.02 acres

Isolation Method employed: Spatial

Volunteers observed: None

Morphological differences noted from non-transgenic: None

Field Trial disposal method, grain: Hand harvest
biomass: plowed under

Increase/decrease in insect susceptibility: No differences noted with the exception of intended ECB tolerance.

Increase/decrease in disease susceptibility: no notable differences

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-108-09N; 95-094-03N

Project: CBH304; CBH305 (96-094-03N); CBH351, CBH354 (96-108-09N)

Planting Site: Aurora, Kane County, IL

Responsible Party:
Marty McGregor
Cargill Hybrid Seeds
2600 W. Galena Blvd.
Aurora, IL 60506
630-801-2325
630-801-2345 (FAX)

Planting Date: May 17, 1996

Harvest Date: October 1, 1996

Number of Transgenic Rows Planted: 55

Total Acreage: 0.05 acres

Isolation Method employed: Spatial

Volunteers observed: None

Morphological differences noted from non-transgenic: None

Field Trial disposal method, grain: Hand harvest
biomass: plowed under

Increase/decrease in insect susceptibility: No differences noted with the exception of intended ECB tolerance.

Increase/decrease in disease susceptibility: no notable differences

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-108-09N

Project: CBH351

Planting Site: Molokai, Maui County, HI

Responsible Party: (please note address change)
Dr. Robert Granger
Cargill Hybrid Seeds
19 Huaai Road
Hoolehua, Molokai, HI 96748
808-567-6871
808-567-9426 (FAX)

Planting Date: November 26, 1996

Harvest Date: March 5, 1997

Number of Transgenic Rows Planted: 94

Total Acreage: 0.10 acres

Isolation Method employed: Temporal

Volunteers observed: None

Morphological differences noted from non-transgenic: None

Field Trial disposal method, grain: Hand harvest
biomass: plowed under

Increase/decrease in insect susceptibility: plants not infested with ECB, no differences noted when compared with non-transgenic materials.

Increase/decrease in disease susceptibility: no notable differences when compared with non-transgenic materials

3 1998

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-108-09N

Project: CBH351

Planting Site: Molokai, Maui County, HI

Responsible Party: (please note address change)
Dr. Robert Granger
Cargill Hybrid Seeds
19 Huaai Road
Hoolehua, Molokai, HI 96748
808-567-6871
808-567-9426 (FAX)

Planting Date: March 22, 1997

Harvest Date: July 1, 1997

Number of Transgenic Rows Planted: 93

Total Acreage: 0.10

Isolation Method employed: Temporal

Volunteers observed: None

Morphological differences noted from non-transgenic: None

Field Trial disposal method, grain: Hand harvest
biomass: plowed under

Increase/decrease in insect susceptibility: plants not infested with ECB, no differences noted when compared with non-transgenic materials.

Increase/decrease in disease susceptibility: no notable differences when compared with non-transgenic materials

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-108-09N

Project: CBH351

Planting Site: Molokai, Maui County, HI

Responsible Party: (please note address change)
Dr. Robert Granger
Cargill Hybrid Seeds
19 Huaai Road
Hoolehua, Molokai, HI 96748
808-567-6871
808-567-9426 (FAX)

Planting Date: July 7, 1997

Harvest Date: September 29, 1997

Number of Transgenic Rows Planted: 26

Total Acreage: 0.03 acres

Isolation Method employed: Temporal

Volunteers observed: None

Morphological differences noted from non-transgenic: None

Field Trial disposal method, grain: Hand harvest
biomass: plowed under

Increase/decrease in insect susceptibility: plants not infested with ECB, no differences noted when compared with non-transgenic materials.

Increase/decrease in disease susceptibility: no notable differences when compared with non-transgenic materials

3 1998

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-108-09N

Project: CBH351

Sites not planted. Nothing to report.

Research Test Site, Champaign County, Illinois: Principal Investigator: Mike Letchworth, 2314 County Road, 1050 N., Homer, IL 61849. phone: 217-688-2361. Up to 1 acre.

Research Test Site, Piatt County, Illinois: Principal Investigator: Mike Letchworth, 2314 County Road, 1050 N., Homer, IL 61849. phone: 217-688-2361. Up to 1 acre.

-- 1996

Annex 8

ENVIRONMENTAL RELEASE REPORT

Permit Number: 96-115-09n

Effective Date: May 24, 1996

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

Transgenic Lines: Corn (*Zea mays*) lines CBH-351, CBH-354

Sites (with maximum acreage) and periods of release:

Champaign County, IL (CBH-354 only, 0.01 acres)	6/96 - 10/96
Maui County, HI (0.1 acres)	8/96 - 5/97

Purpose of Release:

The purpose of the release was to incorporate the transgenic locus into DEKALB germplasm.

Results:

The transgenic locus was incorporated into DEKALB germplasm.

Monitoring of Field Containment:

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

Plant Characteristics 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 field:	no change
Insect resistance:	no change

Any indication that inserted DNA has been transferred to other species, microbial or plant? no

Methods Used to Isolate Transgenic Lines from Other Corn Lines:

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

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

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

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

d) Early termination: Transgenic corn was destroyed before pollen shed.

Means of Plant Disposition:

Plant material was either :

a) stored for future use (e.g. seed for breeding purposes), or
b) destroyed by incorporation into the soil (e.g. repeated disking).

Monitoring for Volunteers:

All volunteers were destroyed prior to anthesis.

Additional Comments:

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

Anne 98

GREAT LAKES

December 1, 1997

Animal and Plant Health Inspection Services
Biotechnology, Biologics, and Environmental Protection
Biotechnology Permits
4700 River Road, Unit 147
Riverdale, MD 20737-1237

Field Trial Termination Report
USDA Notification Number: 96-100-05N

[This document contains confidential business information]

1. Release in Iowa

1.1.	Regulated Article:	[Lines CBH-351 and CBH-354]	CBI
1.2.	Location of trial:	Boone County	
1.3.	Isolation type:	Space	
1.4.	Schedule of trial:	Planting May 30, 1996 Trial End October 26, 1996	
1.5.	Objective of trial:	Efficacy test of BT gene	
1.6.	Transgenic area:	0.015 acres total	
1.7.	General observations:	Plant morphology and growth were similar for both transgenic and non-transgenic plants. No differences in plant phenotype (except for the tolerance to ECB of the transgenic plants), in germination, seedling vigor and disease susceptibility were observed between transgenic and non-transgenic plants. There were no indications for increased weediness of the transgenic plants.	
1.8.	Disposal:	Plant material was chopped and plowed into the field. The area was monitored for volunteers; no volunteer plants were found. The plot area was planted to a rotational crop in spring 1997 (soybeans).	

GREAT LAKES HYBRIDS
9915 West M-21, PO Box 637
Ovid, MI 48866
1-800-257-7333
Fax: 517-725-8356

062

Annex 8

GREAT LAKES

[This document contains confidential business information]

2. Releases in Puerto Rico

2.1.	Regulated Article:	[Lines CBH-304, CBH-305, CBH-351, CBH-354]	CBI
2.2.	Location of trials:	Finca Alomar, Sta. Isabel	
2.3.	Isolation type:	Space, for all trials	
2.4.	Schedule of trials:	1) Planting June 24-28, 1996 Harvest September 19, 1996 2) Planting September 30, 1996 Harvest December 22, 1996 3) Planting November 14, 1996 Harvest February 15, 1997 4) Planting January 14, 1997 Harvest April 10, 1997 5) Planting February 18, 1997 Harvest May 20, 1997 (also under Notification # 97-050-11N)	
2.5.	Objective of trials:	Breeding and observation nurseries	
2.6.	Transgenic area:	Trial 1): 0.2 acres total Trial 2): 0.2 acres total Trial 3): 0.2 acres total Trial 4): 0.2 acres total Trial 5): 0.4 acres total	
2.7.	General observation:	Plant morphology and growth were similar for both transgenic and non-transgenic plants. No differences in plant phenotype, in germination, seedling vigor and disease susceptibility were observed between transgenic and non-transgenic plants. There were no indications for increased weediness of the transgenic plants.	
2.8.	Disposal:	Hand harvest of ears to be further utilized. The remaining plant material was chopped and incorporated into the soil by plowing. For each trial site harvest was followed with a 2 months fallow period during which the trial area was irrigated and monitored for the appearance of corn plants from germinating seeds. Volunteer plants were destroyed by chemical application or by hand.	

Signature Klaus Köhler Date 12-1-97

Klaus Köhler

GREAT LAKES HYBRIDS
9915 West M-21, PO Box 637
Ovid, MI 48866
1-800-257-7333
Fax: 517-725-8356

Annex 8

PLANT GENETIC SYSTEMS

FIELD TRIAL FINAL TERMINATION REPORT

28 October 1997

USDA Notification Number 96-143-03N

Submitted by: Susan MacIntosh
Plant Genetic Systems (America), Inc.
[A wholly owned company of AgrEvo GmbH]

County and State
of Intended Release: Story County, Iowa

Release never occurred. Research agreement between Plant Genetic Systems and Marlin Rice, Ph.D. was never reached, therefore the field trial was delayed until the 1997 season.

PLANT GENETIC SYSTEMS (AMERICA) INC.
7200 Hickman Road • Des Moines, Iowa • 50322 U.S.A.
Phone (515) 276-6642 • Fax (515) 278-8054

3 1998

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-094-16N

Project: CBH305; CBH351; CBH354

Responsible Party:
Marty McGregor
Cargill Hybrid Seeds
2600 W. Galena Blvd.
Aurora, IL 60506
630-801-2325

Planting Date: May 17, 1996

Harvest Date: October 1, 1996

Number of transgenic rows planted: 28 rows

Total Acreage: 0.03 acres

Isolation Method Employed: temporal

Volunteers observed: none

Morphological differences noted from non-transgenic: none

field trial disposal method:
grain: hand harvested; plowed under
biomass: plowed under

Increase/decrease in insect susceptibility:
Efficacy trial - plants infested with European Corn Borer (ECB) larvae. Modified plants showed protection to ECB when compared with the non-modified controls.

Increase/decrease in disease susceptibility: Incidence of stalk rot was reduced in transgenic lines when compared with non-transgenic controls.

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-094-16N

Project: CBH351; CBH354

Responsible Party:
Dr. Hiep Pham
Cargill Hybrid Seeds
2211 6th Avenue
Grinnell, IA 50112
515-236-4911

Planting Date: June 15, 1996

Harvest Date: November 12, 1996

Number of transgenic rows planted: 32

Total Acreage: 0.040 acres

Isolation Method Employed: temporal

Volunteers observed: none

Morphological differences noted from non-transgenic: none

field trial disposal method:
grain: plowed under
biomass: plowed under

Increase/decrease in insect susceptibility:
Efficacy trial - plants infested with European Corn Borer (ECB) larvae. Modified plants showed protection to ECB when compared with the non-modified controls.

Increase/decrease in disease susceptibility: No noted differences when compared with non-transgenic controls.

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-094-16N

Project: CBH351; CBH354

Responsible Party:
Dr. Mark Winkle
Cargill Hybrid Seeds
Bloomington, IL
309-663-2873

Planting Date: June 15, 1996

Harvest Date: week of October 28, 1996

Total Acreage: 0.040 acres

Isolation Method Employed: temporal

Volunteers observed: none

Morphological differences noted from non-transgenic: none

field trial disposal method:
grain: plowed under
biomass: plowed under

Increase/decrease in insect susceptibility:
Efficacy trial - plants infested with European Corn Borer (ECB) larvae. Modified plants showed protection to ECB when compared with the non-modified controls.

Increase/decrease in disease susceptibility: Incidence of stalk rot was reduced in transgenic lines when compared with non-transgenic controls.

Annex 8

FINAL REPORT: CARGILL HYBRID SEEDS

Reference Number: 96-094-16N

Project: CBH305; CBH351; CBH354

Responsible Party:
Dr. Robert Granger
Cargill Hybrid Seeds
Kam Hwy
Kaunakakai, Molokai, HI 96748

Planting Date: No activity to report. Site not planted.

Annex 8

FEB 13 1998

ASGROW.

To:	E.Dianne Hatmaker	**From:**	Clemens de Kok
Fax:	515-232-6905	**Pages:**	3
Phone:	515-232-7170/334	**Date:**	February 12, 1998
Re:	96-102-02N termination report Phoder	**CC:**	Sue Macintosh,Susan Kohler,Arnold

☐ **Urgent** x **For Review** ☐ **Please Comment** ☐ **Please Reply** ☐ **Please Recycle**

• **Comments:** PGS-BT

Attached a copy of the termination report for permit 96-102-02N (Asgrow's request number AG030296.CYS). This report is relevant to petition # 97-265-01P.

Please forward copies to Susan Kohler and Arnold Phoder.Thanks.

Sincerely,

Clemens de Kok

New Concepts Breeder

Annex 8

--- 13 1998

Final report to the USDA -February 12 ,1998

USDA Permit Number : 96-102-02N

Asgrow Permit number : AG030296.CYS

Locations :
Isabela, Puerto Rico
Gibbon, Nebraska
Ames, Iowa
Monmouth, Illinois
Oxford, Indiana
Parkersburg, Iowa
Galena, Maryland
Stonington, Illinois

Note that allthough Parkersburg Iowa and Stonington Illinois are on this permit they were never included in the 1996 PGS trials and or demos that were sent out from Ames.

Responsible researchers:
Dr.Paul Hepperly (PR)
Mr.Dan Lubich (NE)
Mr. Clemens de Kok (IA)
Dr.Kevin Eichelberg (IL)
Dr.Rodney Tietz (IN)
Dr.Greg Holland (IA)
Dr.Mike Hewbaker (MD)
Mr.Eric Beyers (IL)

Transgenic Acreage :
Puerto Rico- .09A
Nebraska - .1A
Iowa - .1A
Illinois - .05A
Indiana - .05A
Maryland - .05A

Dates of release:
Puerto Rico- 6/5/96
Nebraska - 6/4/96
Iowa - 6/3/96
Illinois - 6/5/96
Indiana -6/7 and 6/14/96
Maryland -5/24/96

Isolation method:
Puerto Rico- space isolation
Nebraska -space isolation
Iowa -space isolation
Illinois -time isolation
Indiana -time isolation
Maryland -space isolation

Annex 8

Date of final termination of release:

Puerto Rico	-10/1/96
Nebraska	-10/22/96
Iowa	- 11/10/96
Illinois	-10/28/96
Indiana	-11/10/96
Maryland	-10/18/96

As requested in the supplemental conditions included with the approval of permit # 96-102-02N ,I am submitting a summary of the data collected from the field trial involving our BT tolerant corn as supervised by the above mentioned researchers from Asgrow Seed Co.

The purpose of the trial was to demonstrate Bt gene efficacy in 4 different hybrids .

Means of containement and plant disposition:

All sites were machine planted except Tuscola,Illinois. .Machines were thoroughly cleaned after planting the transgenic material.At all locations left overs of the trial were field incorporated either by plow or disk to minimize volunteers.Fields in all locations were monitored for volunteers rthe season after.In Puerto Rico the field was rotated in soybeans,as well as in Tuscola, Ames, Monmouth and Galena.In Nebraska Poast corn was planted to be sprayed with Poast to elimenate the volunteers.

Disease and Insect susceptibility: No changes were recorded in morphology,disease or insect resistance between the transgenic and non transgenic corn,with the exception of less target insect feeding on the transgenic corn.

Plant growth characteristics and weediness:No differences in seed,seedling or plant growth were noticed that would make the transgenic corn more likely to be a potential weed problem.

Results:The specific event appeared to have a favorable impact on the target insect feeding in different genetic backgrounds,deminishing target insect feeding on the specific hybrids.

Title	MRID#	Date submitted
Characterization of Cry9C and PAT protein levels in Bt Corn under field conditions	44258103	April 4, 1997
Test Substance Characterizations	44258105	April 4, 1997
Determination of Test Substance Equivalence between corn plant produced Cry9C and bacterial produced Cry9C	44384401	Sept. 19, 1997
Insect host range comparison of Cry9C protein	44258106	April 4, 1997
Validation of the determination of Cry9C protein concentration in corn plant powder	44384402	Sept. 19, 1997
An acute oral toxicity study in mice with Cry9C protein as purified from Bacillus thuringiensis Cry9C.PGS2	44258107	April 4, 1997
In vitro digestibility and heat stability of the endotoxin Cry9C protein sequence	44258108	April 4, 1997
Amino acid sequence homology search with the corn expressed truncated Cry9C protein sequence	44258109	April 4, 1997
Environmental Fate Data - Final Report	44161701	Nov. 15, 1996
Chronic exposure of Folsomia candida to corn tissue or bacteria expressing Cry9C protein	44258110	April 4, 1997
Cry9C protein in corn pollen: A dietary toxicity study with the Ladybird beetle (Hippodamia convergens)	44258111	April 4, 1997
Cry9C protein in corn pollen: A 48-hour static-renewal acute toxicity test with the Cladoceran (Daphnia magna)	44258112	April 4, 1997
Cry9C protein in plant powder: An acute toxicity study with the earthworm in an artificial soil substrate	44258113	April 4, 1997
Corn plant powder containing Cry9C protein: A dietary toxicity study with the Northern Bobwhite	44258114	April 4, 1997
Effects of Cry9C corn on Predatory Non-Target Beneficial Insects and Endangered Species	44258115	April 4, 1997
Insect resistance management plan	44258116	April 4, 1997
Preparation and Characterization of Catfish pellets	44384301	Sept. 19, 1997
Cry9C protein in corn pollen: A dietary toxicity study with the honey bee (Apis mellifera)	44384302	Sept. 19, 1997

AgrEvo

97-265-01p

Dr. Susan Koehler
USDA-APHIS, Biotechnology Evaluation
4700 River Road, Unit 147
Riverdale, MD 20737-1236

March 25, 1998

Re: Petition 97-265-01p

Dear Dr. Koehler,

I am glad that the review of AgrEvo's petition is underway. As per our phone conversation on March 5, this letter focuses on Confidential Business Information (CBI) with regard to the AgrEvo petition 97-265-01p. Originally, AgrEvo had requested that certain seed company cooperators remain confidential in order to protect our relationship with them. These circumstances have now changed. Therefore, all information previously declared CBI in the AgrEvo petition 97-265-01p is now allowed to be made public. As you have pointed out, some of the CBI information was either inadvertently included in the CBI-deleted copy of the petition or excluded from the CBI copy of the petition or was only included as part of an original CBI copy of a notification submitted to APHIS for field testing of transformation event CBH-351. The USDA should continue to utilize the CBI-deleted copy of the petition which is currently on public display. The following explanation will clarify where information marked as CBI exists or was omitted from the CBI-deleted copy now on display and discloses as non-confidential that information.

Pages 2 and 37 of the petition list the following USDA notification numbers as CBI: 95-089-04n, 95-094-03n, 95-261-07n, 95-272-08n, 95-284-13n, 95-320-02n, 96-059-02n, 96-075-04n, 96-086-07n, 96-094-16n, 96-100-05n, 96-102-02n, 96-108-09n, 96-115-05n, 96-115-09n. They are no longer declared as CBI.

Annex 8: USDA field trial termination reports
The effected pages and the relevant notification numbers corresponding to the termination reports are listed. Unless otherwise stated, the previously declared CBI is contained in bold within the brackets.

> **Pages 13/14** (95-107-10n): CBI information was only in the original CBI copy of the notification, which was not submitted as part of the CBI-deleted or CBI copy of the Annex 8 of the petition.

AgrEvo USA Company
7200 Hickman Road, Suite 202, Des Moines, IA 50322, Telephone: (515) 276-6642, Fax: (515) 278-8054
Contains no Confidential Business Information

3/27/98
Kay

Page 19 (95-272-08N): CBI information had been deleted from both the CBI-deleted and CBI copy of Annex 8 of the petition. This information is as follows: pRVA9906: [CBH-304, CBH-305] and pRVA9909 + [pDE110] / [CBH-351, CBH-354].

Page 21 (95-272-08N): CBI information had been deleted from both the CBI-deleted and CBI copy of Annex 8 of the petition. This information is as follows: pRVA9909 + [pDE110] and [CBH-351, CBH-354]

Pages 50/51 (96-107-04N): CBI information was only in the original CBI copy of the notification, which was not submitted as part of CBI-deleted or CBI copy of Annex 8 of the petition.

Page 52 (96-115-05N): Both the original CBI-deleted copy and the CBI-copy contained the following bracketed information originally held as CBI by AgrEvo. It is no longer being claimed as CBI. pRVA9906 [CBH-304], pRVA9909 + [pDE110] [CBH-351, CBH-354]

Pages 62/63 (96-100-05N): These pages were not submitted in the original CBI copy, but were submitted to APHIS on February 2, 1998 as additional pages of Annex 8. The pages were not marked as CBI, and therefore they were included in the CBI-deleted copy for public display. These pages contain the following information which is marked as CBI, but which is now not considered by AgrEvo to be CBI. Regulated Article: [Lines CBH-351 and CBH-354]

Please do not hesitate to contact me with any more questions. My phone is (515) 276-6642, fax (515) 278-8054 and email at susan.macintosh@agrevo.com.

Sincerely,

Susan C. MacIntosh

Product Safety Manager

cc: Sally Van Wert
Patrick Rüdelsheim