94-319-019

ciba

Jeffrey Stein
Regulatory Affairs Manager

Ciba Seeds
Agricultural Biotechnology
CIBA-GEIGY Corporation
3054 Cornwallis Road
P.O. Box 12257
Research Triangle Park
North Carolina 27709-2257
Telephone 919-541-8683
FAX 919-541-8585

November 14, 1994

Mr. Michael Lidsky, Esq.
Deputy Director
Biotech Coordination and Technical Assistance
USDA/APHIS
Room 849
Federal Building
6505 Belcrest Road
Hyattsvile, MD 20782

Dear Mr. Lidsky:

On behalf of Ciba Seeds (a division of Ciba-Geigy Corporation), I am pleased to submit a petition to USDA for deregulated status of Ciba Seeds' corn genetically engineered to express a truncated version of the full length CrylA(b) protein that occurs naturally in *Bacillus thuringiensis* subspecies *kurstaki* strain HD-1. This submission provides extensive information and data supporting Ciba Seeds' belief that our transgenic *Bt* corn poses no risk as a plant pest nor a risk to human health or the environment. You will find enclosed two duplicate originals of the petition along with additional reference material requested by USDA, some of which is currently under review at the Environmental Protection Agency as part of an application for registration under FIFRA. The USDA may access additional information presently under review at EPA by requesting such information directly from Ciba Seeds.

We look forward to your earliest possible response on the completeness of this submission. It is our understanding from past discussions with USDA that the lead technical contact on this petition will be David Heron. Please refer all technical comments and questions directly to me at the above telephone or fax number. All broader issues related to policy or interagency coordination should be directed to Rich Lotstein at 910-547-1090.

Thank you in advance for your timely attention to this request. We look forward to working closely with you and your staff.

Sincerely,

Jeffrey Stein
Regulatory Affairs Manager

#1-CS
11,15/94

94-319-01P

PETITION FOR DETERMINATION OF NONREGULATED STATUS OF CIBA SEEDS' CORN GENETICALLY ENGINEERED TO EXPRESS THE CRYIA(b) PROTEIN FROM *Bacillus thuringiensis* subspecies *kurstaki*

The undersigned submits this petition of 7 CFR 340.6 to request that the Director, BBEP, determine that Ciba Seeds' Bt Corn not be regulated under 7 CFR 340.

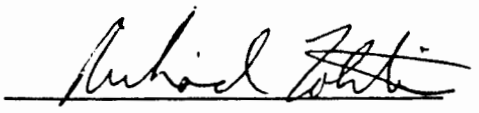
submitted by

Richard Lotstein
Ciba-Geigy Corporation
Ciba Seeds
3054 Cornwallis Road
Research Triangle Park, NC 27709-2257

This Petition Does Not Contain Confidential Business Information

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 relevent data and information known to the petitioner which are unfavorable to the petition



Richard Lotstein
Ciba-Geigy Corporation
Ciba Seeds
3054 Cornwallis Road
Research Triangle Park, NC 27709-2257
1-800-334-9481 Ext. 1090

PETITION FOR DETERMINATION OF NONREGULATED STATUS OF CIBA SEEDS' CORN GENETICALLY ENGINEERED TO EXPRESS THE CRYIA(b) PROTEIN FROM *Bacillus thuringiensis* subspecies *kurstaki*

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CHAPTER 1

Petition for Determination of Non-regulated Status of Ciba Seeds *Bt* Corn

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1. Petition for Determination of Non-regulated Status of Ciba Seeds *Bt* Corn

A. Introduction, Summary and Rationale for Development of *Bt* Corn

Ciba Seeds (a division of Ciba-Geigy Corporation) has developed several lines of corn plants that produce an insect control protein representing a truncated form of the CryIA(b) protein that occurs naturally in *Bacillus thuringiensis* subsp. *kurstaki (B.t.k.)*, a common gram-positive soil bacterium. This protein is a member of a class of insecticidal proteins, also known as delta-endotoxins, that are produced as parasporal crystals by *B. thuringiensis* in nature, and are known to be quite selective in their toxicity to specific organisms, while nontoxic to all other organisms. The crystal proteins are typically produced as large protoxins. Following ingestion by a susceptible insect, the protoxin is solubilized in the alkaline insect gut, and then activated by digestive enzymes to yield a smaller protein. The activated protein binds to specific receptors in the insect midgut and brings about cell lysis by formation of pores. Cessation of feeding and death of the insect follow. Naturally-occurring *B.t.k.* proteins have been commercially produced and used as insecticides for decades. An extensive body of safety testing and experience supports their lack of toxicity to humans and animals, and the absence of adverse effects on nontarget organisms and the environment.

B.t.k. proteins are very effective against certain lepidopteran (caterpillar) insects, including European corn borer (ECB), *Ostrinia nubilalis* (Hubner). This major corn pest reduces yield by disrupting normal plant physiology and causing physical damage to the plant and ear that results in stalk lodging, dropped ears and damaged grain. Two generations of ECB can occur per growing season; adult moths lay eggs on young corn and on fully grown plants. Emerging larvae feed on leaves, leaf sheath tissue, and pollen, and eventually bore into the stalk. Yield reduction due to ECB infestation is estimated to exceed $50 million annually in the state of Illinois alone. ECB damage in the corn belt was especially high in 1990 and 1991, causing up to 30% yield loss in heavily infested fields. Although chemical insecticides such as organophosphates and synthetic pyrethroids as well as B.t. microbial insecticides, can be effective against ECB, applications must be carefully timed before the insect bores into the stalk, and repeat applications are often necessary to achieve control.

The production of a *B.t.k.* insect control protein by corn plants represents a potentially important new option in pest control, and an attractive alternative to external application of insecticides. Results of small-scale field tests by Ciba Seeds under permits granted by the U. S. Department of Agriculture and the Environmental Protection Agency indicate that corn plants producing the CryIA(b) protein are quite effective in controlling ECB, even though only minute quantities are produced. Plant lines under evaluation preferentially express the insect control protein in leaf tissue and pollen (both feeding sources for ECB), while minimizing its production in other plant tissues, including kernels, where it is not needed for control of the target pest.

The *cryIA(b)* gene expressed in the transgenic corn plants encodes a protein that is identical to the first 648 amino acids of the full-length 1155 amino acid CryIA(b) protoxin that occurs in nature. This truncated protein contains the portion of the native protein that is responsible for its insecticidal activity.

In addition to expressing CryIA(b) protein, the plants also express the enzyme phosphinothricin acetyltransferase, currently used in the plant as a selectable marker. This protein product does not confer any pesticidal activity and does not have any

adverse environmental or toxicological effects. It is derived from a ubiquitous, non-pathogenic bacteria, and is readily degraded by proteases.

Large-scale testing was performed in 1993 and 1994 to evaluate the transgenic plants in different geographic areas under a variety of conditions and to cross the insecticidal gene into other corn lines. Activities conducted under USDA permits or notification and an Experimental Use Permit from the EPA accomplished the following objectives through March, 1994:

- Gene efficacy evaluations
- Resistance management experiments
- Insect susceptibility studies
- Breeding
- Seed increases

Activities conducted under USDA and EPA regulations resulted in no detectable adverse environmental impact. Since corn plants in the U. S. do not cross breed with any wild or weedy relatives, the transgenes did not escape to other plant species. The measures employed for containment of seed and pollen ensured that any off-site movement was highly unlikely. Corn is wind-pollinated; the large size of corn pollen and its brief period of viability precluded any significant dissemination beyond the borders of the test plot. Since corn is incapable of surviving in the wild, any unlikely volunteer plants that might have arisen through seed dispersal were incapable of sustained reproduction.

Based on the known mechanism of toxicity of native *B.t.k.* protein, the possibility of any adverse effects on nontarget organisms is extremely remote. Dermal contact by humans does not pose a concern, as the insecticidal protein is contained primarily within the plant material. The potential for dietary exposure of wild birds, mammals or nontarget insect species to transgenic plant tissue has been carefully considered and addressed through extensive testing. Further, the established target-specificity of native *B.t.k.* delta endotoxins supports the lack of toxicity of the CryIA(b) protein to all non-lepidopteran species. Any CryIA(b) protein ingested by non-lepidopterans is expected to be proteolytically inactivated and digested as conventional dietary protein, based on the *in vitro* digestibility studies conducted and reported herein. Likewise, the marker gene product is also expected to be digested in all species as conventional dietary proteins.

Following each field test conducted during the 1992, 1993 and 1994 growing seasons, all plant material not required for future research or plantings was carefully incorporated into the soil to decompose. The minute amount of CryIA(b) protein present was expected to be readily degraded along with the rest of the plant by natural processes. Accordingly, no effects on groundwater or aquatic environments were expected or observed.

Ciba Seeds recognizes the theoretical possibility that both target and non-target insects may eventually develop resistance to maize expressed CryIA(b) protein, as has been known to occur with conventional insecticides (see Reference Document I & Reference Document II). Ciba is working aggressively to avoid or minimize the development of such potential resistance. Some of the field activities were conducted specifically to address this concern. In addition, an intensive research and testing program is underway, both internally and through external collaborators, and through the funding of several university researchers. Ciba's strategy for future product development will likely ensure that additional, effective options in insect control will be available before resistance to the present Bt corn plants could develop.

Transgenic plants hold great promise as an important new tool in integrated pest management programs. This technology allows the crop plant to deliver its own means of protection against insect attack. The expected result is a very specific and directed biological control method that is environmentally sound and that can be expected to reduce the need for manual and chemical inputs by the grower. This, in turn, can have additional environmental and consumer benefits by (1) reducing the risks associated with environmental spills or misapplication of chemical insecticides, (2) eliminating unwanted effects on beneficial insect populations (which can be susceptible to conventional chemical applications); these beneficial insects can, in turn, further reduce the reliance upon chemical means of pest control, (3) reducing the consumption of fossil fuels required to deliver chemical inputs by machinery, and (4) contributing to the availability of a more reliable, high quality and plentiful source of food and feed.

B. Petition to USDA APHIS for Removal of Bt Corn from Regulated Status

This petition is for USDA APHIS to determine that Ciba Seeds' Bt Corn, genetically engineered to produce an insect control protein representing an active portion of the CryIA(b) protein that occurs naturally in *Bacillus thuringiensis* subsp. *kurstaki (B.t.k.)*, as well as an associated marker protein that confers resistance to glufosinate (Ignite® or Basta®) herbicide neither poses a plant pest risk nor has an adverse effect in the environment and should therefore no longer be considered a regulated article under 7 CFR 340. This petition for exemption from 7 CFR 340 covers Ciba transformation event 176 and all progeny of event 176 used in feed and food grade field corn developed and produced by Ciba Seeds as well as industry collaborators of Ciba Seeds who are incorporating the described traits into their own proprietary lines of corn for use in feed and foods such as sweet corn, popcorn, and other traditional corn products.

The only genetic sequences contained in Ciba Seeds' Bt Corn that originate from a plant pest are the cauliflower mosaic virus (CaMV) 35S promoter associated with the *bar* selectable marker gene, and the CaMV terminator associated with the *bar* and Bt genes. The *bar* gene was isolated from *Streptomyces hygroscopicus* which is not a plant pest according to 7 CFR 340.2. Other DNA sequences associated with the plasmid vector have been incorporated into corn, but these are not expressed in plants and originate from *E. coli*, which is not a plant pest according to 7 CFR 340.2.

The recipient organism, *Zea mays*, is not a plant pest according to 7 CFR 340.2.

The Bt gene is a synthetic gene designed for optimal expression in corn and results in expression of a Bt protein 100% homologous to a portion of the CryIA(b) protein that occurs naturally in *Bacillus thuringiensis* subsp. *kurstaki. Bacillus thuringiensis* is not a plant pest according to 7 CFR 340.2.

Two gene promoters and an intron derived from *Zea mays* are used in association with the Bt gene. Transformation was accomplished by use of microprojectile bombardment.

Although CaMV is a regulated article under 7 CFR 340.2, Ciba Seeds' Bt Corn containing sequences from CaMV should not be considered a plant pest risk, as deleterious to the environment, nor as a regulated article for the following reasons:

1. Corn is not a regulated article;
2. Genetic sequences used from CaMV to produce Bt Corn do not code for a specific protein or result in any trait that presents a plant pest or environmental risk;
3. All structural genes introduced into Bt Corn originate from organisms that are not plant pests and do not confer characteristics that would present Bt corn as a plant pest risk;
4. There is no evidence for new compounds in Bt Corn that pose a hazard or are deleterious to the environment.

C. The Corn Family

Excerpted in full from USDA APHIS, Environmental Assessment 92-042-01, pages 6-9, by Dr. James Lackey

1. Corn as a Crop

Zea mays Linnaeus, known as maize throughout most of the world, and as corn in the United States, is a large, annual, monoecious grass, that is grown for animal feed, silage, human grain, vegetable oil, sugar syrups, and other miscellaneous uses. It is the premier cash crop in the United States, and its cultivation, genetics, processing, financing, and distribution on a national and international scale is pervasive and complex.

World production in 1987/1988 was 439 million metric tons, of which the United States produced 179, China 76, Brazil 23, and France 12. Corn is grown commercially in almost all States of the United States (Jewell, 1989). United States production in 1987 was 7064 million bushels, of which the top state producers were Iowa (1306), Illinois (1201), Nebraska (812), Minnesota (635), and Indiana (632). Corn has the highest value of production of any United States crop; 1987 value was 12.1 billion dollars, compared to soybeans at 10.4, hay at 9.1, wheat at 5.4, and cotton at 5.0.

Corn has been cultivated since the earliest historic times from Peru to central North America. The region of origin is now presumed to be Mexico (Gould, 1968). Dispersal to the Old World is generally deemed to have occurred in the sixteenth and seventeenth centuries (Cobley and Steele, 1976); however, recent evidence indicates that dispersal to India may have occurred prior to the twelfth and thirteenth centuries by unknown means (Johannessen and Parker, 1989).

2. Taxonomy of Corn

Zea is a genus of the family Gramineae (Poaceae), commonly known as the grass family. The genus consists of some four species: Z. *mays*, cultivated corn and teosinte; Z. *diploperennis* Iltis et al., diploperennial teosinte; Z. *luxurians* (Durieu et Asch.) Bird; and Z. *perennis* (Hitchc.) Reeves et Mangelsd., perennial teosinte. Various of the species have been assigned to the segregate genus *Euchlaena*, which is not currently recognized, or have been divided into numerous small species within the genus *Zea* (Terrell et al., 1986).

Of the four species of *Zea*, only Z. *mays* is common in the United States. It is known only from cultivation; it occasionally is spontaneous in abandoned fields or roadsides, but is incapable of sustained reproduction outside of cultivation (Gould, 1968). The other species are occasional university or experiment station research subjects. *Zea perennis* is reported as established from James Island, South Carolina (Hitchcock and Chase, 1951).

The closest generic relative to *Zea* is *Tripsacum*, a genus of seven species, three of which occur in the United States (Gould, 1968). *Tripsacum* differs from corn in many respects, including chromosome number (n=9), in contrast to *Zea* (n=10). All species of *Tripsacum* can cross with *Zea*, but only with difficulty and only with extreme sterility (Galinat, 1988).

Cultivated corn is presumed to have been transformed from teosinte, Z. *mays* subspecies *mexicana* (Schrader) Iltis, more than 8000 years ago. During this transformation, cultivated corn gained several valuable agronomic traits, but lost the ability to survive in the wild. Teosinte, however, remains a successful wild grass in Mexico and Guatemala. Despite some confusion over proper taxonomic groupings of the non-cultivated members

of *Zea*, wild members maintain a successful array of annual or perennial plants with visible chromosomal peculiarities and ploidy levels, and many adaptive macroscopic phenotypes. Cultivated corn and the wild members of diploid and tetraploid *Zea* can be crossed to produce fertile F_1 hybrids. Nonetheless, in the wild, introgressive hybridization does not occur because of differences in flowering time, geographic separation, block inheritance, developmental morphology and timing of the reproductive structures, dissemination, and dormancy (Galinat, 1988).

The second major transformation of cultivated corn occurred in the United States in the twentieth century, and particularly since the 1930's. This transformation occurred through inbred lines for hybrid seed production, and by other methods. Almost all corn grown in the United States now comes from hybrid seed that is obtained every planting season from private enterprises; the older open-pollinated varieties are virtually unknown in commerce (Hallauer et al., 1988). This transformation has resulted in more uniform commercial plants with superior agronomic characteristics, and has contributed to the six-fold increase in per acre yields in the last sixty years.

3. Morphology and Reproduction of Corn

Corn is a tall, robust, monoecious annual, with overlapping sheaths and broad, conspicuously distichous blades; staminate spikelets in long spikelike racemes, these numerous, forming large spreading terminal panicles (tassels); pistillate inflorescence in the axils of the leaves, the spikelets in 8-16 (30) rows, on a thickened, almost woody axis (cob), the whole enclosed in numerous large foliaceous bracts or spathes, the long styles (silk) protruding from the summit as a mass of silky threads; grains at maturity greatly exceeding the glumes (Hitchcock and Chase, 1951).

Pollination, fertilization, and caryopsis development of corn follows a fairly standard pattern for chasmogamous wind-pollinated grasses, with the following points of exception and note:

1. Pollen is produced entirely in the staminate inflorescenses. Eggs are produced entirely in the pistillate inflorescences.

2. Self-pollination and fertilization and cross-pollination and fertilization are usually possible and frequencies of each are usually determined by physical proximity and other physical influences on pollen transfer. A number of complicating factors, such as genetic sterility factors and differential growth rates of pollen tubes may also influence the frequencies of self-fertilization versus cross-fertilization.

3. Corn styles and corn pollen tubes are the longest known in the plant kingdom.

4. Shed pollen typically remains viable for 10 to 30 minutes, but may remain viable for much longer under refrigerated conditions (Coe et al., 1988).

5. The staminate and pistillate inflorescences do not develop at the same time. The pistillate inflorescence is precocious. However, there is the appearance of slight protandry because the elongating styles (silks) are delayed for about seven days in emergence from the bracts of the pistillate inflorescence, while the development of the later-developing staminate inflorescence is fully visible. The silks are receptive to pollen up to 10 days after emergence, but receptivity decreases rapidly after that (Walden and Everett, 1961).

6. The genetics of corn is better known than that of any other crop plant.

4. Pollination of Corn

Corn is wind pollinated; insects are responsible for insignificant amounts of pollen dispersal (Russell and Hallauer, 1980). Studies of pollination of corn have mostly centered on the needs of hybrid seed production. This production involves the development and maintenance of inbred lines and the subsequent crosses to produce commercial seed. In the former, self-pollination is mandatory. In the latter, cross-pollination is mandatory. Mechanisms have been developed to ensure each kind of pollination.

Breeder seed is usually derived from self-pollinated seed at the F_8 to F_{10} generation of inbreeding (Wych, 1988). A high degree of self-pollination is ensured by planting well isolated blocks that virtually guarantee natural random sib mating. Minimum isolation distances for foundation seed are one-eighth mile (660 feet) from the nearest contaminating source. Other safeguards, such as physical barriers or unharvested border rows, can further reduce the possibility of contamination. Fields are preferred that have not been recently planted in corn. This is to minimize the appearance of volunteer corn from a previous season.

Hybrid seed production fields also require isolation, similar to that for foundation seed. Isolation distance may be modified by such factors as high winds, additional border rows, size of field, natural barriers, and differential flowering dates. Flowering dates are often adjusted by differential planting dates, planting depth, or fertilizing. The two different parents are planted in a regular pattern of rows, such as four pistillate to one staminate (4:1), or 4:2, or 6:2, or a variety of other combinations. Detasseling or use of cytoplasmic male sterility prevents pistillate plants from shedding viable pollen, and thus ensures cross-pollination.

5. Cultivation of Corn

Corn is grown in the United States as rowcrops of monocultures of uniform plants from hybrid seed. Agronomic practices have developed a high degree of scientific sophistication in the use of tillage, pesticides, planting, fertilizer, harvesting, distribution, and all other agronomic aspects.

6. Weediness of Corn

Corn appears as a volunteer in some fields and roadsides, but it never has been able to establish itself outside of cultivation (Gould, 1968). Some of the other species of *Zea* are successful wild plants, but have no pronounced weedy tendencies (Galinat, 1988).

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CHAPTER 2

The Molecular Biology and Genetic Analysis of Bt Corn

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2. The Molecular Biology and Genetic Analysis of Bt Corn

A. Event 176 Summary

The initial transformation of Ciba's Bt corn was conducted in a propriety corn (*Zea mays* Linnaeus) line, CG00526, an elite inbred of Lancaster parentage. Initial transformants (multiple plants from the single #176 event) in the inbred line CG00526 were crossed to CG00526 as well as to several other elite lines representing several heterotic groups.

The event designated as number 176 has been stably transformed with two plasmids. Transformation vector pCIB4431 (Figure 1) contains two different tissue-specific promoters each fused individually to the synthetic *cryIA(b)* gene. One promoter, the phosphoenolpyruvate carboxylase (PEPC) promoter from corn (Hudspeth and Grula, 1989) confers expression of CryIA(b) protein in green tissue. The second promoter, also isolated from corn, confers protein expression exclusively in pollen (Estruch *et al.*, 1994). The combination of PEPC and pollen tissue-specific promoters provides for relatively high *cryIA(b)* gene expression in leaves and pollen, where it is most effective in controlling European corn borer, while minimizing expression of CryIA(b) protein in kernels and other tissues where it is not needed.

The second vector used in the transformation of event #176, pCIB3064 (Figure 2), harbors a gene that confers resistance to phosphinothricin, the active ingredient in the herbicide glufosinate.

Transgenic corn plants derived from transformation event 176 have been evaluated under USDA permits 92-042-01, 92-127-01r, 92-140-01r and 93-014-01r, 93-363-01N, 94-056-06N, 94-076-10N, and EPA Experimental Use Permit #66736-EUP-1.

B. Description of the Transformation, Selection and Regeneration Protocols Used to Create Event 176

Transformation was achieved through microprojectile bombardment (reviewed by Sanford, 1990) of immature embryos (excised 14-15 days after pollination) using a PDS-1000He Biolistic™device (for detailed methodology, refer to Koziel *et al.*, 1993). Plasmids pCIB4431 and pCIB3064 were co-transformed. No additional DNA (e.g., carrier DNA) was used during the transformation process.

One day after bombardment, embryos were transferred to callus initiation medium containing phosphinothricin (PPT) as a selective agent. Resultant embryogenic tissue was cultured in callus maintenance medium containing PPT for 12 weeks, and then transferred to regeneration medium. After several more weeks in culture, transformed plants were identified using the chlorophenol red assay (Kramer *et al.*, 1992) to test for resistance to PPT, and PCR analysis for specific sequences in the 35S promoter and the synthetic *cryIA(b)* gene.

Plants positive in both analyses were transferred to the greenhouse for additional testing and crossing with various inbreds. To minimize the time required to obtain hybrid progeny plants for small-scale field testing (under USDA permits), immature embryos (14-16 days after pollination) were removed and cultured as described in Weymann *et al.*, 1993. Insecticidal activity was confirmed by bioassay using European corn borer larvae. CryIA(b) protein levels in the transgenic plants were determined by ELISA; these data are described elsewhere in this submission (Table 3A)

C. Descriptions of Gene Constructs Used in the Transformation Process

Profiles for the two plasmid constructs used in transformation event 176 are given below.

1. Gene Profile for pCIB4431

This plasmid is comprised of the two tissue specific promoters, each fused to an identical *cryIA(b)* gene, cloned into the vector pUC19 (Figure 1; Yanisch-Perron *et al*, 1985).

- **phosphoenolpyruvate carboxylase promoter**

This promoter, 2.32 Kb in length, is derived from the corn phosphoenolpyruvate carboxylase gene described in Hudspeth and Grula, 1989. In our transgenic maize plants, it promotes expression of *cryIA(b)* in green tissue. This sequence does not encode for any protein(s).

- **synthetic *cryIA(b)* gene**

This 1.94 Kb fragment of DNA encodes a truncated version of the full-length *cryIA(b)* gene occurring in *Bacillus thuringiensis* subsp. *kurstaki* strain HD-1 (Dulmage, 1970; Geiser *et al.*, 1986; Hofte and Whiteley, 1989). It encodes the first 648 amino acids of this protein, which has been shown to be the insecticidal-active region (Koziel *et al*, 1993). The entire coding region of this gene has been synthesized in order to increase its G+C content, thereby accommodating the preferred codon usage for maize (Murray *et al.*, 1989). These changes result in an enhanced level of expression of the *cryIA(b)* gene in plants (Perlak *et al.*, 1991; Koziel *et al.*, 1993). However, there has been no alteration to the amino acid coding sequence of the *cryIA(b)* gene. It is 100% identical in content and sequence, at the amino acid level, to the native CryIA(b) protein (Fig 8). The native gene has a G+C content of about 38% while the synthetic version is about 65%.

- **PEPC intron #9**

This 0.11 Kb fragment of DNA contains the number 9 intervening sequence from the corn phosphoenolpyruvate carboxylase gene (Hudspeth and Gura, 1989). This DNA sequence is located between the *cryIA(b)* structural gene and the 35S terminator. Its presence in the plasmid is to increase the expression level of the *cryIA(b)* gene. The expression level of genes in plants has been shown to be enhanced by the presence of introns (Luehrsen and Walbot, 1991). This sequence does not code for any protein(s).

- **CaMV 35S terminator**

This 160 bp sequence is from the cauliflower mosaic virus (CaMV) genome. It is present twice in this plasmid, in each case adjacent to the PEPC intron #9. Its function is to provide a polyadenylation site, and has been described previously (Rothstein *et al.*, 1987; Sanfacon *et al.*, 1991). This sequence does not code for any protein(s).

- **pollen-specific promoter**

This promoter, 1.49 Kb in length, is derived from a maize calcium-dependent protein kinase (CDPK) gene that is exclusively expressed in pollen (Estruch *et al.*, 1994). This sequence does not code for any protein(s). The activity of this promoter, associated with its native CDPK structural gene in maize, is not modulated by calcium levels in the plant. Rather, the catalytic activity of the mature CDPK protein in maize is affected by calcium levels. Therefore, we do not anticipate that fusion of this calcium-independent promoter sequence to the *cryIA(b)* gene will manifest in any changes in the calcium requirements of the plant cell.

- **synthetic *cryIA(b)* gene (#2)**

This gene is identical to that fused to the PEPC promoter as described previously in this section of the document.

- **PEPC Intron #9**

It is identical to the PEPC intron #9 associated with the PEPC fusion described earlier in this section of the document.

- **CaMV 35S terminator**

It is identical to the CaMV 35S terminator associated with the PEPC fusion described earlier in this section of the document.

2. Gene Profile for pCIB3064

This plasmid is comprised of the 35S-promoter from cauliflower mosaic virus fused to the plant selectable marker gene *bar* from *Streptomyces hygroscopicus,* cloned into the vector pUC19 (Figure 2).

- **CaMV 35S promoter**

This promoter, 0.64 kb in length, is derived from CaMV genome (Odell *et al.*, 1985; Rothstein *et al.*, 1987). This sequence does not code for any protein(s). It was used to promote expression of the plant selectable marker gene *bar*.

- ***bar* Gene**

This gene, 0.6 Kb in length, encodes the enzyme phosphinothricin acetyltransferase (PAT) which inactivates phosphinothricin (PPT), the active component in glufosinate (De Block *et al.*, 1987; Thompson *et al.*, 1987). The natural substrate for PAT is bialaphos, the naturally produced antibiotic from the same actinomycete from which the *bar* gene was isolated, *Streptomyces hygroscopicus*. There are no other reported substrates for PAT, nor has PAT demonstrated any activity towards other acetyltransferase substrates (Bell and Charlwood, 1980). Expression of this enzyme allows for selection of transformed plant cells on selective medium, as well as whole-plant tolerance to glufosinate application.

- **CaMV 35S terminator**

It is identical to the CaMV 35S terminator associated with the PEPC fusion described earlier in this section of the document.

The plasmid vector into which the sequences detailed above have been cloned, is pUC19. Plasmid pUC19 also harbors 1) a beta-lactamase (*bla;* 0.79 kb) gene under the control of a promoter known only to be active in bacterial cells. This gene confers ampicillin resistance to the bacterium; 2) the ColE1 origin (0.52 kb) that permits replication of pUC19 in bacteria, 3) a 0.32 kb sequence which codes for the alpha-peptide of the beta-galactosidase(*lacZ*) gene. Other than these described regions, there are no known proteins encoded by the remaining DNA sequences in pUC19 (Covarrubias *et al.*, 1981; Yanisch-Perron *et al.*, 1985).

D. DNA Analysis of Transformation Event 176

Southern blot analysis using genomic DNA and element specific probes was performed to determine the nature of the insertion in event #176.

1. *cryIA(b)* analysis

Southern blot analysis was performed using event #176 and control genomic DNA which had been digested with either HindIII, EcoRI, or double digested with HindIII and EcoRI. Blots were hybridized with ^{32}P-labeled *cryIA(b)* coding region. For reconstruction analysis the plasmid pCIB4431 was also digested with the same enzyme(s) and run on the same gel as a control. HindIII cuts once while EcoRI cuts twice in the vector pCIB4431 (Figure 1). Using HindIII digested DNA, for each copy of pCIB4431 present in the genome we predict two hybridizing bands, since HindIII cuts between the two *cryIA(b)* genes in pCIB4431. As depicted in Figure 3, a HindIII digest (lane 4) of event #176 DNA generated four bands that hybridized to the *cryIA(b)* probe. This would indicate at least two copies of this transformation vector present in the genome. This interpretation was supported by the EcoRI data (lane 6), where the presence of four hybridizing bands also indicates at least two copies of the plasmid. The restriction fragments predicted for the plasmid control are also seen in the reconstruction lanes (7-12). There was no hybridization of the *cryIA(b)* gene probe to the untransformed control line CG00526 (lanes 1-3).

2. *bar* analysis

A Southern blot, prepared in the same fashion as described for *cryIA(b)* , was probed with the ^{32}P-labeled *bar* coding region. As shown in Figure 4, the restriction fragments predicted for vector pCIB3064 (Figure 2) are present in the reconstruction lanes (7-12). No hybridization is seen in control line CG00526 (lanes 2 and 3). Hybridization to two bands in the HindIII/EcoRI double digest indicate at least two copies of the *bar* gene are present in event 176. These bands also correspond, as expected, to restriction fragments which hybridize with a CaMV 35S promoter specific probe (Figure 6, lane 5).

3. CaMV 35S promoter analysis

A Southern blot prepared in the same fashion as described for *cryIA(b)* was probed with ^{32}P-labeled 35S promoter fragment. As shown in Figure 6 the restriction fragments predicted for plasmid pCIB3064 are seen in the reconstruction lanes (7-12). As expected, the hybridization pattern for event 176 (lanes 4-6) with this 35S-probe is identical to that seen with the *bar* coding region specific probe (Figure 4). This hybridization pattern indicates at least two copies of CaMV 35S promoter-*bar* gene fusion are present in line 176. No hybridization is seen in the control line CG00526 (lanes 2, 3).

4. amp (*bla*) analysis

A Southern blot prepared in the same fashion as described for *cryIA(b)* was probed with ^{32}P-labeled *bla* gene. One copy of this gene is present in both transformation vectors. In Figure 5, the restriction fragments predicted for both plasmids (Figures 2, 3) are seen in the reconstruction lanes (7-12). Event 176 (lanes 4-6) shows multiple hybridizing bands. These bands correspond to DNA fragments that also hybridize to either the *cryIA(b)* probe or the *bar* gene probe. This indicates that these genes are proximal to one another in the genome. No hybridization is seen in the control line CG00526 (lanes 2, 3).

E. Insertion Site Analysis and Genetics of Event 176

Genetic evaluation of Ciba Seeds' transformation Event 176 indicates a dominant Mendellian inheritance pattern for both transgenic traits, glufosinate tolerance and protection against European corn borer. Evidence to support the hypothesis that the transgenes in event 176 act as a single effective insertion has been obtained from segregation analyses and from RFLP (restriction fragment length polymorphism) mapping studies aimed at localizing the insertion point in the maize genome.

As an integral part of Ciba Seeds' initial efforts to introgress Event 176 transgenes into its elite maize breeding lines and in the development of inbred lines that are homozygous for the transgenes, Ciba Seeds monitored the segregation of the transgenic traits. Populations of plants were evaluated for glufosinate tolerance (conferred by the *bar* gene), protection against first brood larvae of European corn borer (ECB1) (conferred by the *cryIA(b)* gene), and Southern analyzed using the *cryIA(b)* gene as probe. The results from a typical analysis are presented in Figure 7. All plants from this first-backcross population (BC1) that hybridized to the *cryIA(b)* probe were also bioactive in the ECB bioassay and tolerant to glufosinate. Plants sensitive to glufosinate were inactive in the ECB bioassay and failed to hybridize to the *cryIA(b)* probe. This type of analysis was extended to greater than 3200 plants representing several backcross 1, backcross 2, and F2 populations. The segregation analyses of these populations indicate that glufosinate tolerance and ECB tolerance cosegregate as tightly linked Mendellian traits. Further, the *cryIA(b)* hybridization pattern of the parental event 176 plant (Figure 7, lane 1) and *cryIA(b)*-harboring plants from this BC1 population is indistinguishable. This has been the observation regardless of the elite breeding line used for introgression of the *cryIA(b)* gene. On occasion, some of the plants (< 4 %) exhibit minor changes from the standard observed banding pattern. These differences have been observed in progeny of both hemizygous and homozygous transgenic plants, and represent only molecular weight changes within the standard pattern, not segregation of bands to different progeny. Western analyses have revealed no differences in the molecular weight of the CryIA(b) protein, regardless of the DNA banding pattern. One polymorphic pattern (0.2% frequency) results in loss of expression of the protein from leaf tissue. This occurrence is well within the established industry standards for seed purity.

In order to map the insertion point of the transgenes within the maize genome, 2 BC1 populations of Event 176 consisting of 62 and 59 plants were treated with glufosinate and individual plants were scored for sensitivity or tolerance to the herbicide. DNA extracted from leaf samples taken from the tolerant plants was probed with RFLP probes with known map locations in the corn genome. Subsequent segregation and linkage analyses conducted to determine the location of the insertion point indicated that the *bar* gene(s) act as a single locus and map between the RFLP probes CG320 and CG378 on Chromosome 1. When the same plant DNA was probed with the *cryIA(b)* gene as an RFLP probe, the resulting linkage analysis (MAPMAKER/QTL Version 1.1; 1993) indicated that the *cryIA(b)* gene(s) map to the same chromosomal location as the *bar* gene(s).

This analysis has since been extended through four backcross generations, and the results from these studies confirms the mapping data generated from the initial studies: the *cryIA(b)* and *bar* genes map to the same chromosomal location.

The glufosinate tolerance trait has been used as a selectable marker in Ciba Seeds' breeding work, and it may be one component of a quality control program for foundation and commercial seed production. Following purity checks of approximately 3240 hybrid plants in 1993 field trials, 5 plants (0.15%) were identified as tolerant to glufosinate and susceptible to ECB. This low percentage of apparent recombinants is further evidence of the tight linkage of the transgenic traits. The occurrence of less than 0.2% variants which are ECB susceptible is well within established industry standards for seed purity and will have negligible effect on product performance.

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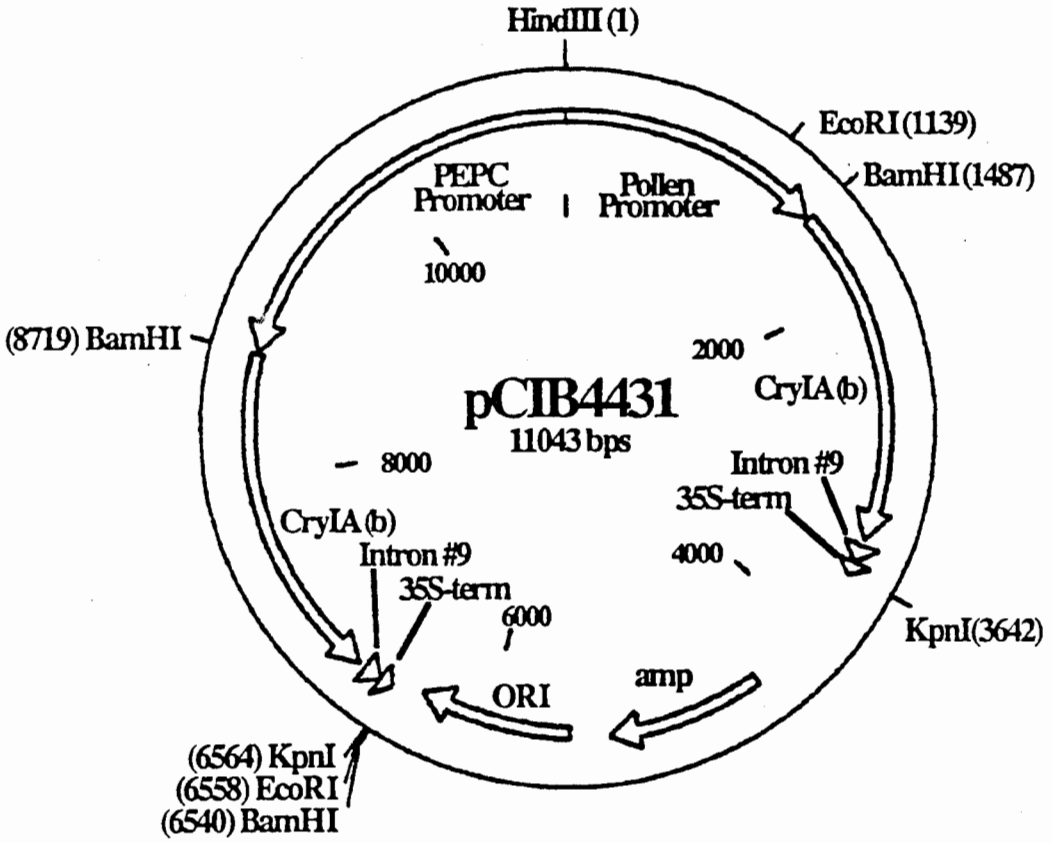

Figure 1. Plasmid pCIB4431
pCIB4431 is a pUC-derived plasmid containing two copies of the synthetic *crylA(b)* gene, one copy under the control of the PEPC promoter, and the other under the control of the maize-derived pollen-specific promoter. Both genes have the PEPC intron #9 and CaMV 35S termination sequence in the 3' untranslated region. Selected endonuclease sites are indicated; ORI: plasmid origin of replication; amp: beta-lactamase (*bla*) gene.

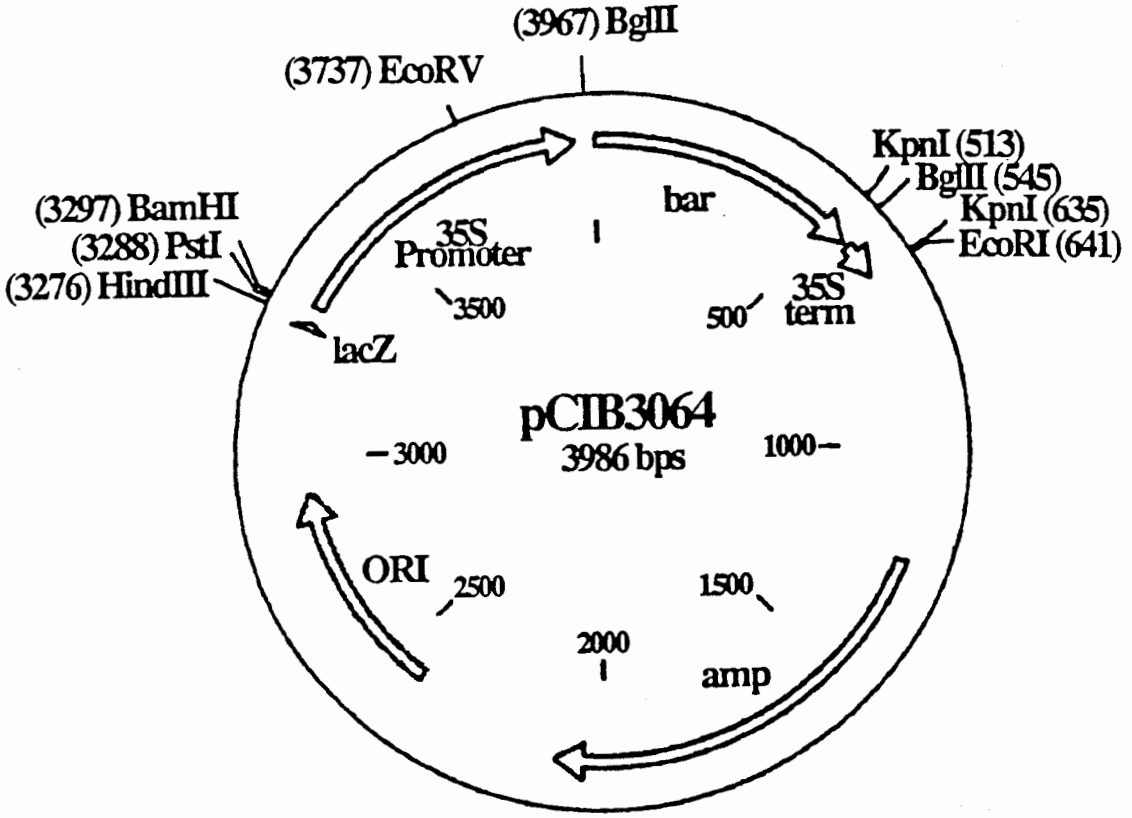

Figure 2. Plasmid pCIB3064
pCIB3064 is a pUC-derived plasmid containing the *bar* gene (encoding phosphinothricin acetyltransferase) under the control of the cauliflower mosaic virus 35S promoter, and 35S termination sequence. Selected endonuclease sites are indicated; ORI: plasmid origin of replication; amp: beta-lactamase (*bla*) gene.

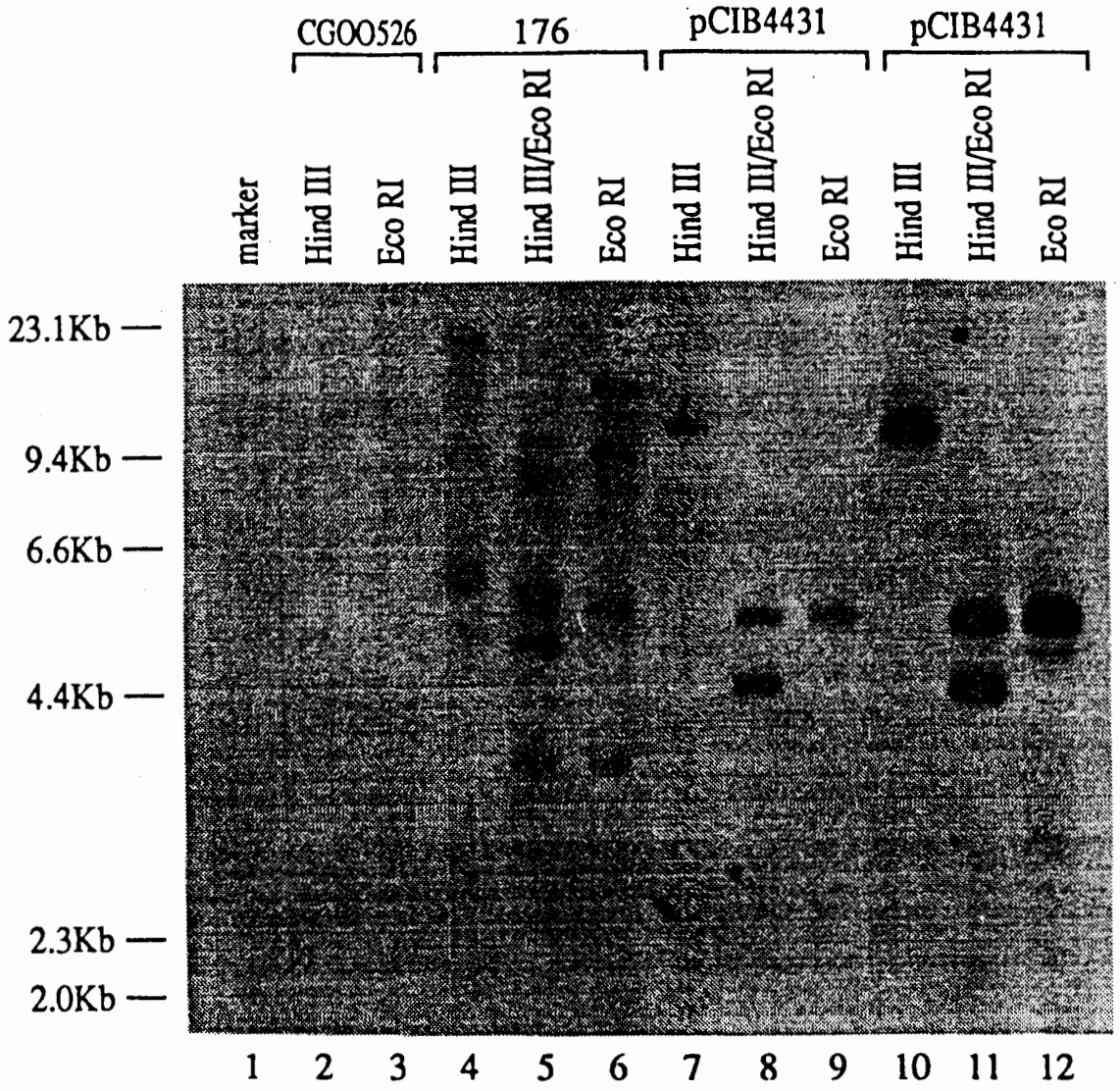

Figure 3. Southern Analysis for *crylA(b)* Gene Insertion
Southern blot analysis to determine the number of insertions of pCIB4431. 10 μg of genomic DNA from control line CG00526 was digested with the enzyme HindIII (lane 2), and EcoRI (lane 3), and10 μg of genomic DNA from event 176 was digested with HindIII (lane 4), HindIII and EcoRI (lane 5) and EcoRI (lane 6). Reconstructions of plasmid pCIB4431 digested with HindIII (lanes 7, 10), HindIII and EcoRI (lanes 8, 11), and EcoRI (lanes 9, 12); 5 pg of plasmid DNA was loaded in lanes 7, 8 and 9, and 25 pg of plasmid DNA was loaded in lanes 10, 11 and 12. Digested DNA was separated by electrophoresis on 0.5% agarose gels, transferred to nylon membrane and probed with ^{32}P-labeled *crylA(b)* coding region.

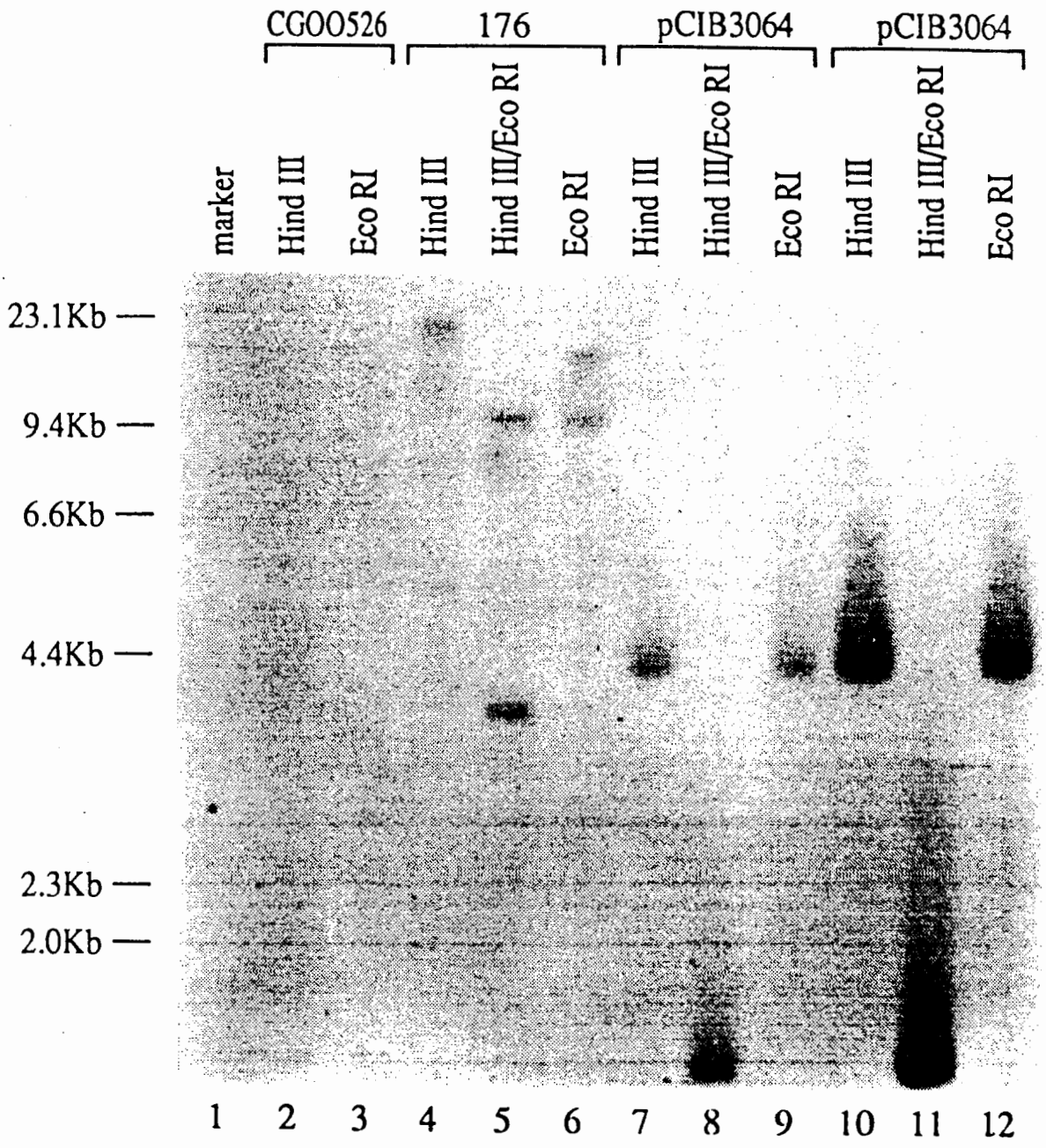

Figure 4. Southern Analysis for *bar* Gene Insertion
Southern blot analysis to determine the number of insertions of the *bar* gene. 10 μg of genomic DNA from control line CG00526 was digested with the enzyme HindIII (lane 2), and EcoRI (lane 3); 10 μg of genomic DNA from line 176 was digested with HindIII (lane 4), HindIII and EcoRI (lane 5), and EcoRI (lane 6). Reconstructions of plasmid pCIB3064 digested with HindIII (lanes 7, 10), HindIII and EcoRI (lanes 8, 11), and EcoRI (lanes 9, 12); 5 pg of plasmid DNA was loaded in lanes 7, 8 and 9, and 25 pg of plasmid was loaded in lanes 10, 11 and 12. Digested DNA was separated by electrophoresis on 0.5% agarose gels, transfered to nylon membrane and probed with ^{32}P-labeled *bar* coding region.

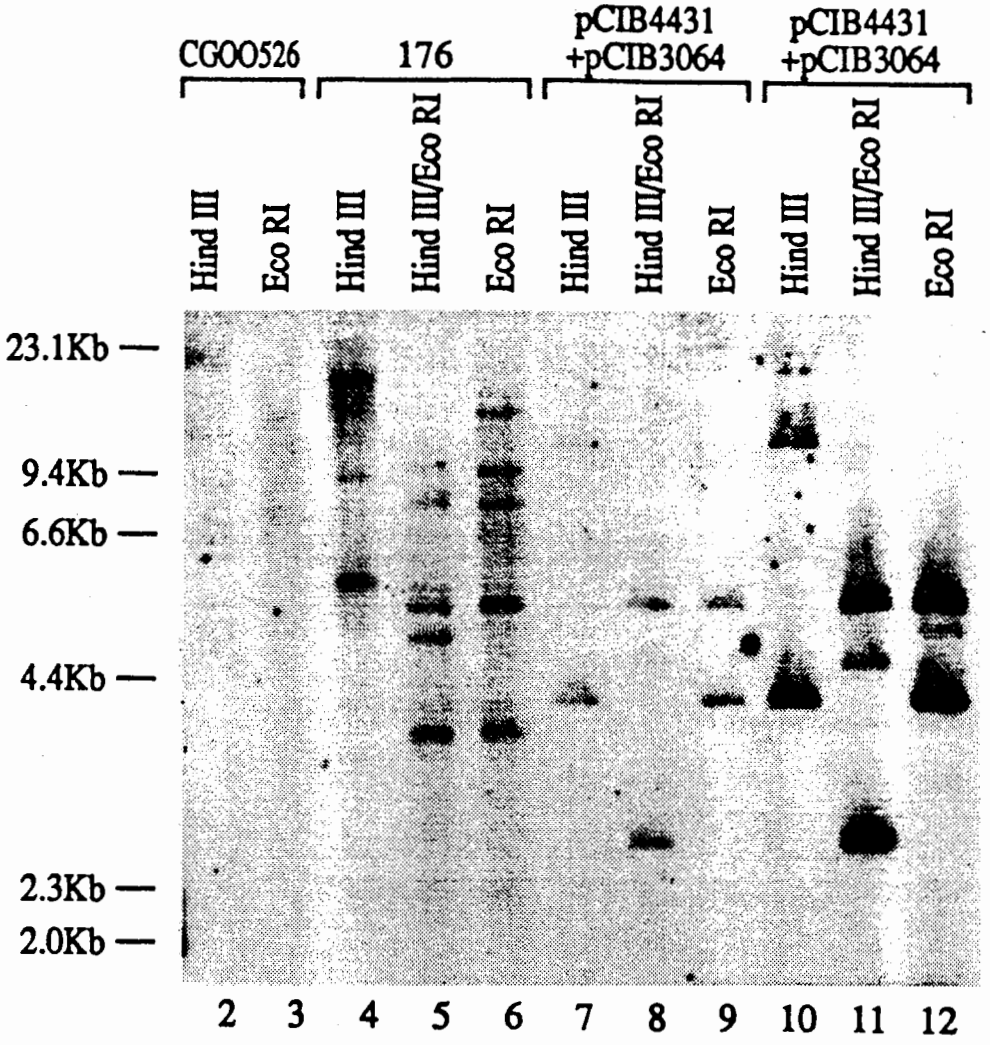

Figure 5. Southern Analysis for amp^R gene Insertion

Southern blot analysis to determine the number of plasmid derived amp^R (*bla*)gene insertions. 10 µg of genomic DNA from control line CG00526 was digested with the enzyme HindIII (lane 2), and EcoRI (lane 3); 10 µg of genomic DNA from line 176 was digested with HindIII (lane 4), HindIII and EcoRI (lane 5), and EcoRI (lane 6). Reconstructions of plasmids pCIB4431 and pCIB3064 digested with HindIII (lanes 7, 10), HindIII and EcoRI (lanes 8, 11), and EcoRI (lanes 9, 12); 5 pg of each plasmid was loaded in lanes 7, 8 and 9, and 25 pg of each plasmid was loaded in lanes 10, 11 and 12. Digested DNA was separated by electrophoresis on 0.5% agarose gels, transfered to nylon membrane and probed with ^{32}P-labeled *bla* gene.

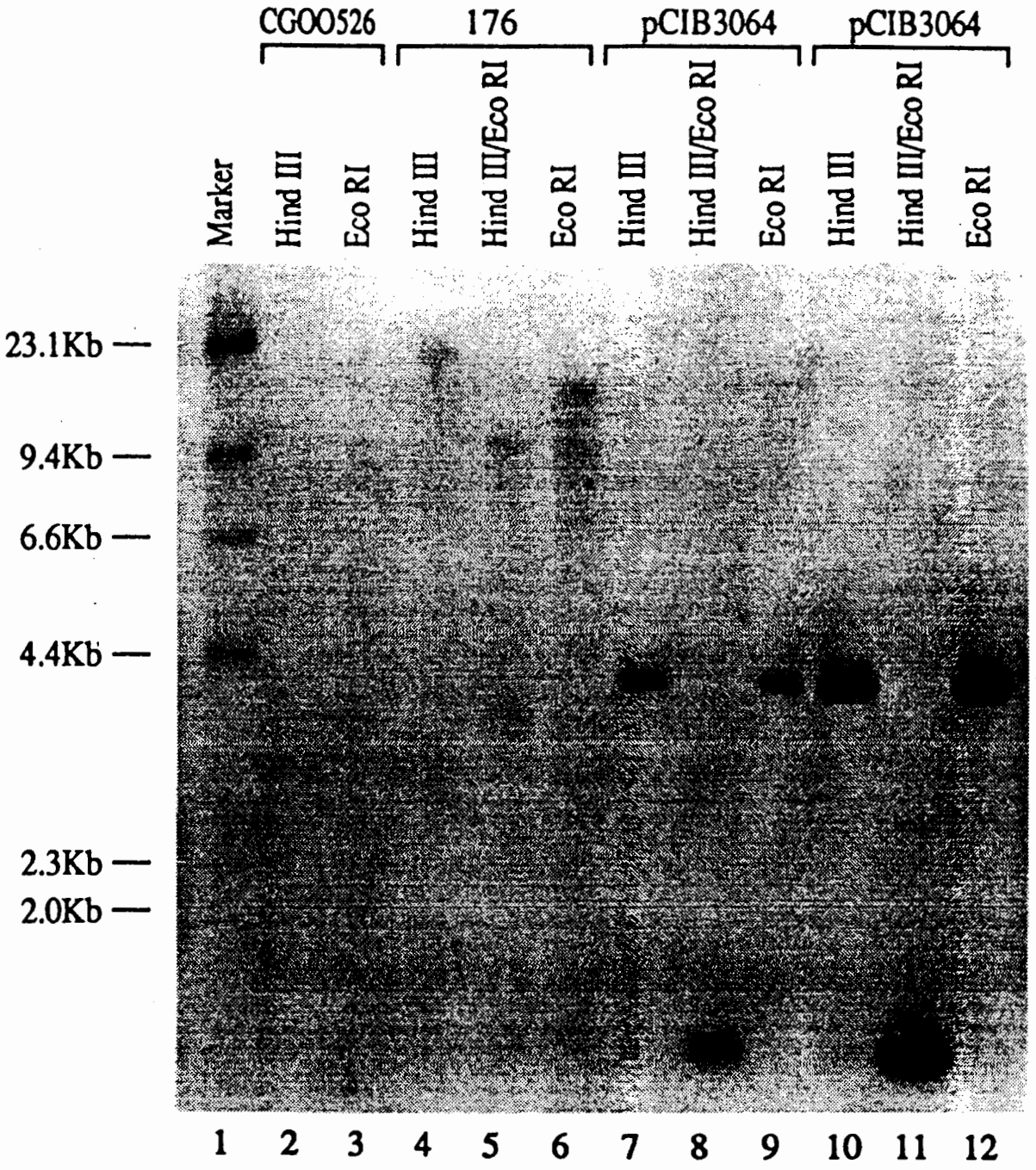

Figure 6. Southern Analysis for CaMV 35S Promoter Insertion
Southern blot analysis to determine the number of CaMV 35S promoter elements present. 10 μg of genomic DNA from control line CG00526 was digested with the enzyme HindIII (lane 2), and EcoRI (lane 3); 10 μg of genomic DNA from event 176 was digested with HindIII (lane 4), HindIII and EcoRI (lane 5), and EcoRI (lane 6). Reconstructions of plasmid pCIB3064 digested with HindIII (lanes 7, 10), HindIII and EcoRI (lanes 8, 11), and EcoRI (lanes 9, 12); 5 pg of plasmid was loaded in lanes 7, 8 and 9, and 25 pg of plasmid was loaded in lanes 10, 11 and 12. Digested DNA was separated by electrophoresis on 0.5% agarose gels, transfered to nylon membrane and probed with ^{32}P-labeled CaMV 35S promoter.

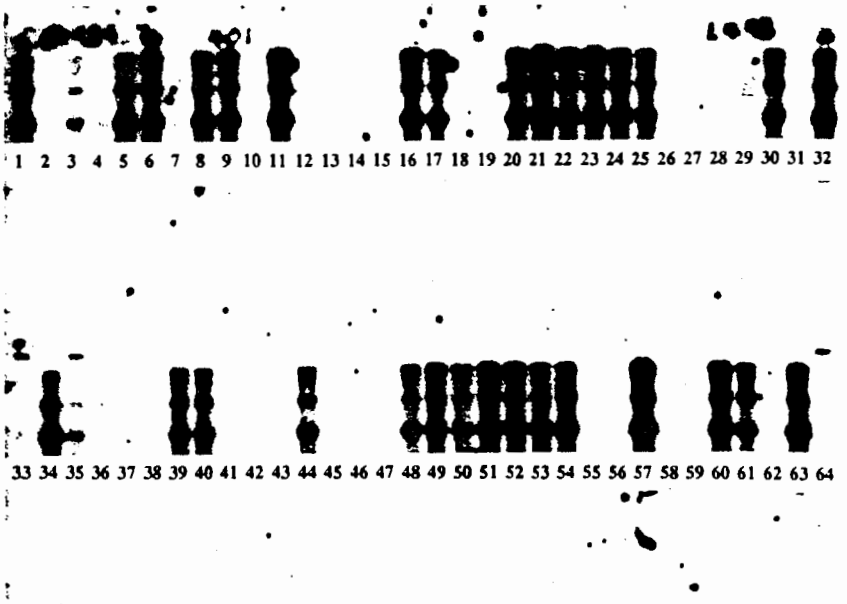

Figure 7. Southern Analysis for *CryIA(b)* Insertion in a Backcross Population of Event 176-Derived Maize Plants

Southern blot analysis of 62 individuals from a first backcross population (CG00554 X CG00526-176). 10 µg of genomic DNA was digested with HindIII and separated by electrophoresis on a 0.5% agarose gel. Following transfer to nylon membrane, the blot was probed with ^{32}P-labelled *cryIA(b)* coding region. Lane 1: CG00526-176 parent; Lane 2: empty; Lanes 3-64: progeny from backcross population.

Figure 8. Amino Acid sequence of the native, full-length CryIA(B) protein [designated 'CryIA(B)-pro'], and the truncated CryIA(b) protein representative of the synthetic gene (designated 'synpro').

```
                    10        20        30        40        50        60
                     *         *         *         *         *         *
CRYIA(B).PRO   MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         MDNNPNINECIPYNCLSNPEVEVLGGERIETGYTPIDISLSLTQFLLSEFVPGAGFVLGL

                    70        80        90       100       110       120
                     *         *         *         *         *         *
CRYIA(B).PRO   VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         VDIIWGIFGPSQWDAFLVQIEQLINQRIEEFARNQAISRLEGLSNLYQIYAESFREWEAD

                   130       140       150       160       170       180
                     *         *         *         *         *         *
CRYIA(B).PRO   PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         PTNPALREEMRIQFNDMNSALTTAIPLFAVQNYQVPLLSVYVQAANLHLSVLRDVSVFGQ

                   190       200       210       220       230       240
                     *         *         *         *         *         *
CRYIA(B).PRO   RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         RWGFDAATINSRYNDLTRLIGNYTDHAVRWYNTGLERVWGPDSRDWIRYNQFRRELTLTV

                   250       260       270       280       290       300
                     *         *         *         *         *         *
CRYIA(B).PRO   LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         LDIVSLFPNYDSRTYPIRTVSQLTREIYTNPVLENFDGSFRGSAQGIEGSIRSPHLMDIL

                   310       320       330       340       350       360
                     *         *         *         *         *         *
CRYIA(B).PRO   NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         NSITIYTDAHRGEYYWSGHQIMASPVGFSGPEFTFPLYGTMGNAAPQQRIVAQLGQGVYR

                   370       380       390       400       410       420
                     *         *         *         *         *         *
CRYIA(B).PRO   TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         TLSSTLYRRPFNIGINNQQLSVLDGTEFAYGTSSNLPSAVYRKSGTVDSLDEIPPQNNNV

                   430       440       450       460       470       480
                     *         *         *         *         *         *
CRYIA(B).PRO   PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         PPRQGFSHRLSHVSMFRSGFSNSSVSIIRAPMFSWIHRSAEFNNIIPSSQITQIPLTKST
                                         :

                   490       500       510       520       530       540
                     *         *         *         *         *         *
CRYIA(B).PRO   NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         NLGSGTSVVKGPGFTGGDILRRTSPGQISTLRVNITAPLSQRYRVRIRYASTTNLQFHTS

                   550       560       570       580       590       600
                     *         *         *         *         *         *
CRYIA(B).PRO   IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID
               ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
synpro         IDGRPINQGNFSATMSSGSNLQSGSFRTVGFTTPFNFSNGSSVFTLSAHVFNSGNEVYID
```

Figure 8 (continued). Amino Acid sequence of the native, full-length CryIA(B) protein [designated 'CryIA(B)-pro'], and the truncated CryIA(b) protein representative of the synthetic gene (designated 'synpro').

```
                    610       620       630       640       650       660
                     *         *         *         *         *         *
CRYIA(B).PRO    RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQVSNLVECLSDEFC
                ::::::::::::::::::::::::::::::::::::::::::::::::::
synpro          RIEFVPAEVTFEAEYDLERAQKAVNELFTSSNQIGLKTDVTDYHIDQV----------

                    670       680       690       700       710       720
                     *         *         *         *         *         *
CRYIA(B).PRO    LDEKKELSEKVKHAKRLSDERNLLQDPNFRGINRQLDRGWRGSTDITIQGGDDVFKENYV
synpro          ----------------------------------------------------------

                    730       740       750       760       770       780
                     *         *         *         *         *         *
CRYIA(B).PRO    TLLGTFDECYPTYLYQKIDESKLKAYTRYQLRGYIEDSQDLEIYLIRYNAKHETVNVPGT
synpro          ----------------------------------------------------------

                    790       800       810       820       830       840
                     *         *         *         *         *         *
CRYIA(B).PRO    GSLWPLSAPSPIGKCAHHSHHFSLDIDVGCTDLNEDLGVWVIFKIKTQDGHARLGNLEFL
synpro          ----------------------------------------------------------

                    850       860       870       880       890       900
                     *         *         *         *         *         *
CRYIA(B).PRO    EEKPLVGEALARVKRAEKKWRDKREKLEWETNIVYKEAKESVDALFVNSQYDRLQADTNI
synpro          ----------------------------------------------------------

                    910       920       930       940       950       960
                     *         *         *         *         *         *
CRYIA(B).PRO    AMIHAADKRVHSIREAYLPELSVIPGVNAAIFEELEGRIFTAFSLYDARNVIKNGDFNNG
synpro          ----------------------------------------------------------

                    970       980       990      1000      1010      1020
                     *         *         *         *         *         *
CRYIA(B).PRO    LSCWNVKGHVDVEEQNNHRSVLVVPEWEAEVSQEVRVCPGRGYILRVTAYKEGYGEGCVT
synpro          ----------------------------------------------------------

                   1030      1040      1050      1060      1070      1080
                     *         *         *         *         *         *
CRYIA(B).PRO    IHEIENNTDELKFSNCVEEEVYPNNTVTCNDYTATQEEYEGTYTSRNRGYDGAYESNSSV
synpro          ----------------------------------------------------------

                   1090      1100      1110      1120      1130      1140
                     *         *         *         *         *         *
CRYIA(B).PRO    PADYASAYEEKAYTDGRRDNPCESNRGYGDYTPLPAGYVTKELEYFPETDKVWIEIGETE
synpro          ----------------------------------------------------------

                   1150
                     *
CRYIA(B).PRO    GTFIVDSVELLLMEE
synpro          ---------------
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CHAPTER 3

Qualitative Analysis of CryIA(b) Expression in Maize Tissue

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3. Qualitative Analysis of CryIA(b) Expression in Maize Tissue

A. Summary

CryIA(b) protein extracted from leaves and pollen of *Bt* maize Event 176 was found to have the predicted molecular weight of approximately 65,000, and to be immunoreactive with antibodies specific for native *B.t.k.* crystal protein. N-terminal sequence analysis of CryIA(b) from *Bt* maize leaves revealed the predicted amino acid sequence with an N-terminus at the position of glycine-25 encoded by *cryIA(b)*. Additional immunoreactive polypeptides corresponding to molecular weights of ca. 60,000, 40,000 and 36,000 were also detected in *Bt* maize leaf extracts but not in pollen extracts. These lower molecular weight proteins appeared to be degradation products of CryIA(b) resulting from intrinsic proteolysis in leaves. Within the detection limits of the available methods, no evidence was found of post-translational modifications such as acetylation, glycosylation or phosphorylation of maize-expressed CryIA(b). Biological activity (LC_{50}) comparisons of maize-expressed and native CryIA(b) among several lepidopterous insect species demonstrated the same rank order of sensitivity to CryIA(b) protein among the susceptible species, and confirmed the absence of insecticidal activity against the insensitive species.

B. Introduction

The truncated CryIA(b) protein (ca. 65,000 mol. wt.) encoded by the synthetic *cryIA(b)* gene is identical in amino acid sequence to the N-terminal 648 amino acids of the 1155 amino acid full-length native CryIA(b) protein produced by *B.t.k.* strain HD1 (Figure 1). The 648 amino acids encoded by the synthetic *cryIA(b)* gene include the portion of the native protein necessary for insecticidal activity. The truncated CryIA(b) expressed in maize, like the native CryIA(b) protein, undergoes proteolytic cleavage in the alkaline gut of a susceptible lepidopterous insect to yield the active insecticidal protein. This lepidopteran-active protein is approximately 564 - 578 amino acids in length (ca. 60,000 mol. wt.) (Höfte *et al.*, 1986).

In order to demonstrate that maize-expressed CryIA(b) in plants derived from Event 176 is substantially similar to native CryIA(b) produced in the bacterium, CryIA(b) protein was extracted from the leaves of *Bt* corn plants and compared biochemically and functionally with the native CryIA(b) protein produced by *B.t.k.* The two proteins were biochemically compared for:

a) protein size
b) immunoreactivity
c) N-terminal amino acid sequence
d) post-translational modifications including:
    - acetylation,
    - glycosylation
    - phosphorylation

Selected additional analyses of CryIA(b) protein from maize pollen were also conducted to evaluate protein size and immunoreactivity. Biological comparisons were made by evaluating the relative sensitivity of various lepidopteran insect species to both maize-expressed CryIA(b) protein and native CryIA(b) protein.

C. Material and Methods

CryIA(b) Protein Size and Immunoreactivity. To compare molecular weight and immunoreactivity of maize-expressed CryIA(b) with native CryIA(b) the following materials were analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and western blot:

Test Material	Prepared as described in
CryIA(b)-enriched *Bt* maize leaf protein	Appendix 3A
CryIA(b) from *Bt* maize pollen	Appendix 3B
Full length native CryIA(b)	Appendix 3C
The major tryptic fragment of CryIA(b)	Appendix 3C

Samples were prepared for SDS-PAGE by addition of an equal volume of 2X Laemmli sample buffer (20% glycerol, 2% b-mercaptoethanol, 4% SDS, 0.13 M Tris-HCl, 0.05% bromophenol blue, pH 6.8). After heating for 10 min at 65°C, the samples were subjected to SDS-PAGE on 10% polyacrylamide gel (Novex, San Diego, CA). The proteins were electroblotted onto Immobilon-P® membrane (Millipore, Bedford, MA). The blots were developed using immunoaffinity-purified rabbit antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 at a concentration of 0.133 mg antibody/ml blocking buffer (3% nonfat dry milk, 10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5). Goat anti-rabbit IgG linked to horseradish peroxidase (Bio-Rad, Hercules, CA), diluted 1:3000 in blocking buffer, was used to bind to the primary antibody and was detected by development with the chromogenic substrate diaminobenzidine (Harlow and Lane, 1988). Western blots of pollen samples were developed using the chemiluminescent substrate in an ECL™ kit (Amersham, Arlington Heights, IL).

N-terminal Amino Acid Sequence Analysis. N-terminal amino acid sequence analysis was performed to determine whether the CryIA(b) protein produced in maize had the predicted amino acid sequence encoded by the synthetic *cryIA(b)* gene with which the maize was transformed (Figure 1). Immunopurified maize CryIA(b) (Appendix 3A) was concentrated and desalted for N-terminal sequence analysis by vacuum blotting onto Immobilon-P® and washing twice with 0.5 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 7.5. The concentrated proteins were removed from the membrane using 30 ml ProBlott Extraction buffer (62 mM Tris-HCl, 6 M urea, 2% b-mercaptoethanol, 10% glycerol, 2% Triton X-100, pH 6.8) (ABI applications brochure #42, Applied Biosystems, Foster City, CA). After the addition of 5 ml of 10 X Laemmli sample buffer (50% glycerol, 10% b-mercaptoethanol, 20% SDS, 0.65M Tris-HCl, 0.25% bromophenol blue, pH 6.8), the sample was heated at 65°C for 10 min and subjected to SDS-PAGE on a 10% polyacrylamide gel (Novex, San Diego, CA). The proteins were electroblotted onto ProBlott™ (Applied Biosystems, Foster City, CA) (Matsudaira, 1987) and stained with 0.1% Amido black. Bands migrating at positions corresponding in molecular weight to maize CryIA(b) (ca. 65,000) and a putative degradation product (ca. 36,000) were cut out and subjected to automated Edman degradation with an Applied Biosystems model 476 pulsed-liquid phase protein sequencer (Strickler *et al.*, 1984) to derive N-terminal amino acid sequence. The phenylthiohydantoin (PTH) amino acid derivatives were separated and identified with an on-line analyzer fitted with a phenylthiohydantoin C_{18} column which was calibrated with PTH amino acid standards prior to each run.

Post-Translational Modifications.

The method of choice for examining maize-expressed CryIA(b) for post-translational modifications was mass spectroscopy. However, technical difficulties (i.e., solvent incompatibility and solubility limitations, purity of sample, etc.) and limiting quantities of purified maize-expressed CryIA(b) prohibited this approach. Therefore, the methods described below were utilized.

Post-translational Acetylation Studies. Many eucaryotic proteins carry an N-acetyl group added post-translationally to their N-terminal amino acid (Tsunasawa and Sakiyama, 1984). In addition, histone proteins are highly acetylated on the e-amino group of specific lysines (Georgieva *et al.*, 1991). Although acetylation of proteins is rare in bacteria (Tsunasawa and Sakiyama, 1984) and therefore not likely to occur in the native CryIA(b) protein, it was of interest to determine whether CryIA(b) protein expressed in maize was subject to post-translational acetylation. N-terminal sequence analysis provided evidence that there was no acetylation at the N-terminal residue (see **RESULTS; N-terminal sequence**). There are only 5 lysine residues (designated K on Figure 1), and therefore only 5 potential acetylation sites among the 648 amino acids in the protein encoded by the synthetic *cryIA(b)* gene expressed in maize. Amino acid sequencing has shown that lysines which are subject to acetylation reside in highly basic domains and that highly specific acetyltransferases are required for acetylation (Georgieva, *et al.*, 1991 and Lopez-Rodas, *et al.*, 1991). The region in which the lysine residues occur in CryIA(b) is not highly basic, i.e. relatively free of histidine, arginine and additional lysine residues (Figure 1), and therefore unlikely to serve as a substrate for an acetyltransferase.

Post-translational Glycosylation Studies. Glycosylation is a common post-translational modification that occurs during the protein secretory process leading to specific compartmentalization of some proteins (Chrispeels, 1991). The information necessary for targeting to specific compartments or organelles within eucaryotic cells resides in specific signals or domains within the primary amino acid sequence of the protein as encoded by its gene. Protein glycosylation occurs in the Golgi apparatus to which the protein must be directed after exiting the endoplasmic reticulum. The synthetic *cryIA(b)* construct does not contain this kind of sequence information which would automatically target it into the secretory pathway. However, the amino acid sequence of maize-expressed CryIA(b) contains six potential N-glycosylation consensus sites (Figure 1). In addition, O-glycosylation can potentially occur at any serine or threonine residue (designated S and T, respectively, in Figure 1) of which there are more than 100 encoded by the synthetic *cryIA(b)* gene. To verify that these sites were not post-translationally glycosylated in maize the Glycotrack® detection system was used.

This procedure involves an amplified periodic acid-Schiff method to reveal carbohydrate moieties on electroblotted proteins. Both immunopurified maize CryIA(b) and full length native CryIA(b) were evaluated by this procedure. Control maize leaf protein (Appendix 3A) was analyzed for background plant non-CryIA(b) glycoproteins. SDS-PAGE was performed as described above (see **CryIA(b) protein size and immunoreactivity**); duplicate gels were run. The proteins were electroblotted onto Immobilon-P® (Millipore, Bedford, MA). One blot was developed as described for western blot analysis using ECL™ kit (see **CryIA(b) protein size and immunoreactivity section**) to detect the position of CryIA(b) bands. On the second blot, carbohydrate moieties associated with the transferred proteins were detected using the Glycotrack® kit (Oxford Glycosystems, Rosedale, NY) according to the manufacturer's instructions. This procedure involves biotinylating carbohydrate residues hydrolyzed by periodic acid and then detection of the biotin by streptavidin conjugated to alkaline phosphatase. Ovalbumin was the

glycoprotein standard used in these studies. The level of sensitivity of this method was determined experimentally to be between 0.1 and 0.2 mg of ovalbumin, which is 5% carbohydrate by weight, or approximately 12 glycosyl (mol. wt. 180) residues per ovalbumin molecule, ca. 45,000 mol. wt. This would correspond to approximately 18 glycosyl residues per molecule of maize-expressed CryIA(b), ca. 65,000 mol. wt..

Post-translational Phosphorylation Studies. Protein phosphorylation is catalyzed by protein kinases which recognize specific amino acid sequences in proteins. Phosphorylation is generally considered to be a regulatory mechanism in the signal transduction process by which the phosphorylation state of a protein corresponds to its state of activity (Randall and Blevins, 1990). Only one protein kinase in maize, a calcium dependent protein kinase (CDPK), has been well characterized (Estruch *et al.*, 1994). Sequence analysis of the CryIA(b) protein expressed in maize revealed only one CDPK consensus phosphorylation site (-RXXS or T-; where R is arginine, X is any amino acid, S is serine, and T is threonine) (Robert and Harmon, 1992) at amino acids 506 - 509 (RTTS) (Figure 1). This was the only CDPK consensus site present in the tryptic fragment of native CryIA(b) as well. Other potential phosphorylation sites exist in the maize CryIA(b) amino acid sequence (i.e. 13 casein kinase II consensus sites, -S or TXXD or E-; where S is serine, T is threonine, X is any amino acid, D is aspartate, and E is glutamate) as identified by the MOTIFS function of the University of Wisconsin Genetics Computer Group Program, version 7.1. There are, however, no maize kinases available for testing phosphorylation of CryIA(b) at these other sites. Therefore, ^{32}P-radiolabeling experiments were designed to determine if the CDPK consensus site in the tryptic fragment of native CryIA(b) was a potential phosphorylation site for maize CDPK. From this, it could be inferred whether maize CryIA(b) could also serve as a substrate for maize CDPK.

Using a maize CDPK provided by Dr. J. Estruch (Estruch *et al.*, 1994), attempts were made to phosphorylate the tryptic fragment of native CryIA(b). ^{32}P-labeling of a CDPK synthetic peptide substrate, which contains two phosphorylation sites (CDPK II fragment 290-309, Cat. No. C-4926, Sigma, St. Louis, MO), was used as a positive control. The complete reaction mix, in a volume of 25 µl, contained 250 ng (6 mM) of the tryptic fragment of native CryIA(b), 50 mM HEPES, 5 mM $MgCl_2$, 30 mM ATP with 240 cpm/pmole ∂-^{32}P-ATP (Amersham, Arlington Heights, IL), pH 7.2, and 50 ng CDPK (Estruch *et al.*, 1994). Controls were prepared identically with the omission of CDPK, and nonradioactive reactions were prepared without the addition of ∂-^{32}P-ATP. As a positive control, a synthetic CDPK substrate was used at a concentration of 200 mM and the reaction volume was doubled. After 10 min at 37°C reactions containing the tryptic fragment of native CryIA(b) were terminated by addition of 5 µl 10X Laemmli sample buffer and heated for 10 min at 95°C. Duplicate samples were subjected to SDS-PAGE on parallel 10% polyacrylamide gels followed by either autoradiography to detect radiolabeling or by western blot analysis to confirm that the CryIA(b) retained its integrity during the treatment. Reactions containing the synthetic CDPK substrate were terminated by adding 500 µl 20% trichloroacetic acid (TCA) in 0.2% sodium pyrophosphate. Samples were collected on 0.22 mm GS filters (Millipore, Bedford, MA), washed with 10 ml 20% TCA and analyzed by scintillation counting using a LS500TA scintillation counter (Beckman, Fullerton, CA).

Biological Activity Comparison of Maize-expressed and Native CryIA(b). To confirm there was no difference in the host-range activity spectrum of maize-expressed CryIA(b) and native CryIA(b), a series of tests was conducted in which six lepidopterous insect species were bioassayed with CryIA(b) protein from either CryIA(b)-enriched maize leaf protein or cell paste from a culture of *B.t.k.* strain HD1-9. Complete dose-

response lines were generated for four CryIA(b)-sensitive insects: European corn borer (*Ostrinia nubilalis*), corn earworm (*Helicoverpa zea*), cabbage looper (*Trichoplusia ni*), and southwestern corn borer (*Diatraea grandiosella*). Two CryIA(b)-insensitive insects, fall armyworm (*Spodoptera frugiperda*) and black cutworm (*Agrotis ipsilon*), were exposed to single doses corresponding to the European corn borer LC_{90} for CryIA(b).

D. Results

Protein Size and Immunoreactivity. Maize-expressed CryIA(b) (ca. 65,000 mol. wt.) from leaf, full length native CryIA(b) (ca. 128,000 mol. wt.), and the tryptic fragment of native CryIA(b) (ca. 60,000 mol. wt.) were subjected to SDS-PAGE followed by western blot analysis. The predicted differences in molecular weights were observed (Figure 2). The full length native CryIA(b) preparation showed multiple immunoreactive bands with the two major bands corresponding to the full-length protein of ca. 128,000 mol. wt. and a lower molecular weight band at ca. 60,000 mol. wt. The tryptic fragment of native CryIA(b) showed only the ca. 60,000 mol. wt. band as anticipated for this polypeptide. The bands forming the doublet at ca. 65,000 mol. wt. in the CryIA(b)-enriched maize leaf protein sample correspond to the predicted size of the 648 amino acid truncated CryIA(b) protein with and without the first 24 - 28 amino acids. Three other bands of lesser intensity were observed at approximate molecular weights of 60,000, 40,000, and 36,000.

The 36,000 mol. wt. band appeared to be the predominant degradation product in several different preparations. Despite inclusion of protease inhibitors in the extraction buffer, similar apparent proteolytic fragments were also present in crude leaf extracts (data not shown). *Bt* maize leaf extracts prepared under denaturing conditions that preclude proteolysis also showed these polypeptides. Moreover, when the tryptic fragment of native CryIA(b) was spiked into a control maize leaf extract prepared without protease inhibitors, it was recovered in an undegraded form (data not shown). Western blot analysis of CryIA(b) from maize leaves of other transformation events also revealed similar-sized immunoreactive bands (data not shown). On the basis of these and other observations, it appears likely that the polypeptides below 65,000 mol. wt. arose through degradation of CryIA(b) by intrinsic maize leaf proteases, and were neither artifacts of extraction nor encoded by *cryIA(b)* genes which had been randomly fragmented during the transformation process. As described by Höfte *et al.* (1986), the minimum-sized CryIA(b) fragment retaining lepidopteran insecticidal activity is approximately 564 - 578 amino acids (Figure 1), corresponding to ca. 60,000 mol. wt. Therefore it is expected that any immunoreactive polypeptides of less than 60,000 mol. wt. present in *Bt* maize leaf protein extracts do not contribute to insecticidal activity. No immunoreactive material was detected in the control maize leaf protein preparation.

No degradation of CryIA(b) protein was apparent in the *Bt* maize pollen. A single band of immunoreactive material was visualized at ca. 65,000 mol. wt., corresponding to the predicted size of the (648 amino acid) protein encoded by the *cryIA(b)* transgene (Figure 3). As indicated by the absence of lower mol. wt. bands, pollen appears to be free of the protease activity exhibited by maize leaf tissue. No immunoreactive material was detected in control nontransgenic pollen (Figure 3).

N-terminal Sequence Analysis. N-terminal amino acid sequence analysis of the two major immunoreactive maize leaf CryIA(b) proteins, after purification by immunoaffinity chromatography and SDS-PAGE, indicated that the ca. 65,000 mol. wt. protein began at the 25th amino acid, glycine, encoded by both the native and synthetic *cryIA(b)* genes. The detected sequence corresponded identically to the predicted sequence over the

subsequent amino acids for which sequence was obtained (10 amino acids total). The N-terminal amino acid of the ca. 36,000 mol. wt. polypeptide corresponded to the 31st amino acid, threonine, of the native CryIA(b) protein and showed complete sequence identity over the subsequent amino acids for which sequence was obtained (15 amino acids total). This identified the 36,000 mol. wt. protein as an N-terminal fragment that extends to approximately amino acid 350 - 400 encoded by the native and synthetic *cryIA(b)* genes, as estimated by its size (Figures 1 and 4).

CryIA(b) expressed in maize appears to be susceptible to intrinsic maize leaf proteases which produce fragments similar to those seen for native CryIA(b) (Figure 2). Native CryIA(b) is susceptible to loss of the N-terminal 28 amino acids, with no loss of bioactivity (Höfte et al., 1986). Sufficient material was not available to conduct N-terminal sequence analysis of the CryIA(b) protein produced in pollen. However, western blot analysis suggests that the single immunoreactive band observed for this tissue corresponds to the predicted size of ca. 65,000 mol. wt (Figure 3).

Acetylation Studies. N-terminal sequence analysis showed that the N-terminal amino acid of maize CryIA(b) was not blocked, i.e., not acetylated. Acetylation of internal lysines has not been ruled out but is deemed unlikely due to the non-basic environment surrounding these residues in CryIA(b) and the specificity of the acetyltransferases required for these modifications (Georgieva *et al.*, 1991, and Lopez-Rodas *et al.*, 1991).

Glycosylation Studies. Maize-expressed CryIA(b) and full length native CryIA(b) were evaluated for post-translation glycosylation using the Glycotrack® procedure, for which the level of sensitivity was approximately 18 glycosyl residues per maize-expressed CryIA(b) molecule (Figure 5). Due to limiting amounts of immunopurified maize CryIA(b) available for analysis, these experiments were conducted under conditions approaching the limit of detection of the procedure. CryIA(b) contains 6 potential N-glycosylation sites as well as over 100 serine or threonine residues, any of which may be glycosylated. Each site in turn can have multiple glycosyl residues associated with it. Under the analysis conditions described here, no glycosylation was detected but the possibility cannot be completely ruled out that up to approximately 18 glycosyl residues may have been undetectable by the methods used.

Phosphorylation Studies. Under the conditions used, radiolabelling of a CDPK synthetic peptide substrate containing two phosphorylation sites was demonstrated to incorporate ^{32}P at a level of ca. 680,000 cpm. If the single CDPK phosphorylation site had become phosphorylated in the tryptic fragment of CryIA(b), an estimated 10,000 cpm would have been incorporated. If CryIA(b) were phosphorylated with only 50% of the efficiency of the synthetic peptide, the estimated 5000 cpm that would have been incorporated should have been sufficient to give a positive signal during autoradiography. No radiolabeling of the tryptic fragment of native CryIA(b) protein was detected by autoradiography after SDS-PAGE (Figure 6). These results indicate that under these conditions the tryptic fragment of native CryIA(b) was not phosphorylated by maize CDPK. Therefore, it is reasonable to conclude that maize CryIA(b) protein is not phosphorylated by CDPK. As discussed in Materials and Methods (see **Post-translational phosphorylation studies**), phosphorylation of maize CryIA(b) by other maize protein kinases cannot be ruled out.

Biological Activity Comparison of Maize-expressed and Native CryIA(b). Under the conditions of this study, no change in the host-range activity spectrum of native CryIA(b) was detected with CryIA(b) extracted from *Bt* maize (Table 1). Among species for which reliable LC_{50}s were determined, the same rank order of sensitivity was observed, i.e., European corn borer was the most sensitive to CryIA(b) followed by cabbage looper and then corn earworm. For most species, mean LC_{50} values for native CryIA(b) ranged from 5.2 to 10.2-fold higher than the corresponding plant CryIA(b) mean LC_{50} values. A reliable LC_{50} for maize CryIA(b) could not be determined for southwestern corn borer, due to apparent feeding inhibition by both the *Bt* maize and control maize leaf protein materials in this species.

It was predicted by composition that the maize CryIA(b) would appear more potent in the insect bioassays than native CryIA(b); this arises from the fact that the 564 - 578 amino acid active insecticidal polypeptide represents ca. 90% of the (648 amino acid) maize CryIA(b) by weight, whereas it represents only ca. 50% of the native (1155 amino acid) CryIA(b) polypeptide by weight (Figure 1). The apparent difference in potency beyond the expected 2:1 ratio may arise from various factors, including the fact that CryIA(b) could not be quantified using the same method for both preparations. Therefore, strict quantitative comparisons between the LC_{50} values derived for maize and native CryIA(b) are not possible; qualitative comparisons of the two CryIA(b) materials indicate that the relative sensitivity among CryIA(b) sensitive insects has not changed.

No increased sensitivity to the plant-derived CryIA(b) was detected for fall armyworm and black cutworm, suggesting that the host-range has not expanded to include these relatively insensitive species.

A more detailed description, including methodology, of this study is presented in Chapter 11 of this report entitled "Environment Safety Data".

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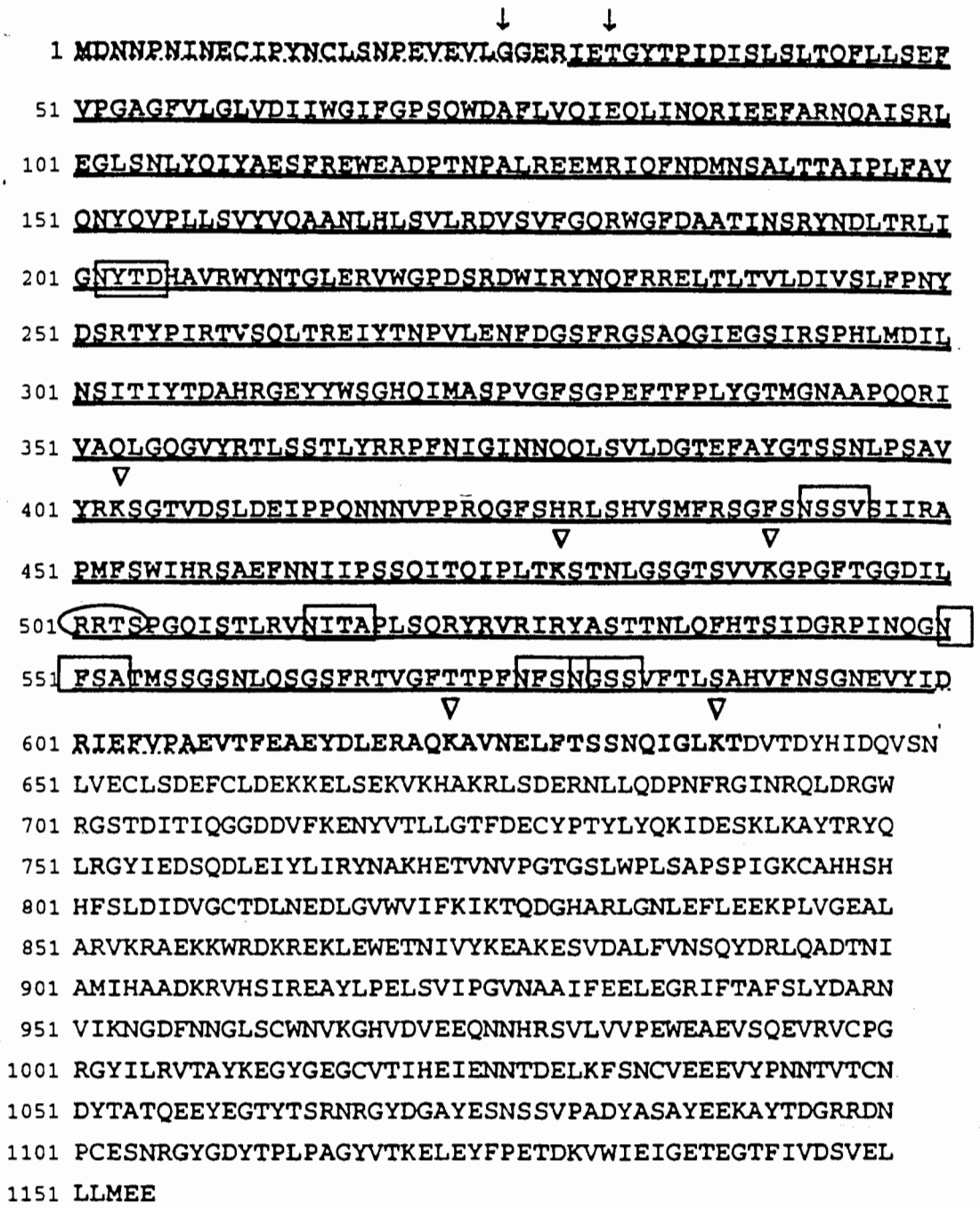

Figure 1. Amino acid sequence of synthetic *cryIA(b)* and native *cryIA(b)* products. The amino acid sequence encoded by the synthetic *cryIA(b)* gene transformed into maize is indicated in bold (1-648). The native gene encodes amino acids 1-1155 shown above. The arrows indicate the N-termini (G-25 and T-31) of the major CryIA(b) polypeptides found in *Bt* maize. The rectangles enclose N-glycosylation consensus sequences (see Text); the ellipse encloses the potential CDPK phosphorylation site. The ∇ marks the five lysines present in the maize-expressed gene. The minimal insecticidal fragment is underlined. The major tryptic fragment of native CryIA(b) includes the minimal fragment together with that which is underscored by the dotted line.

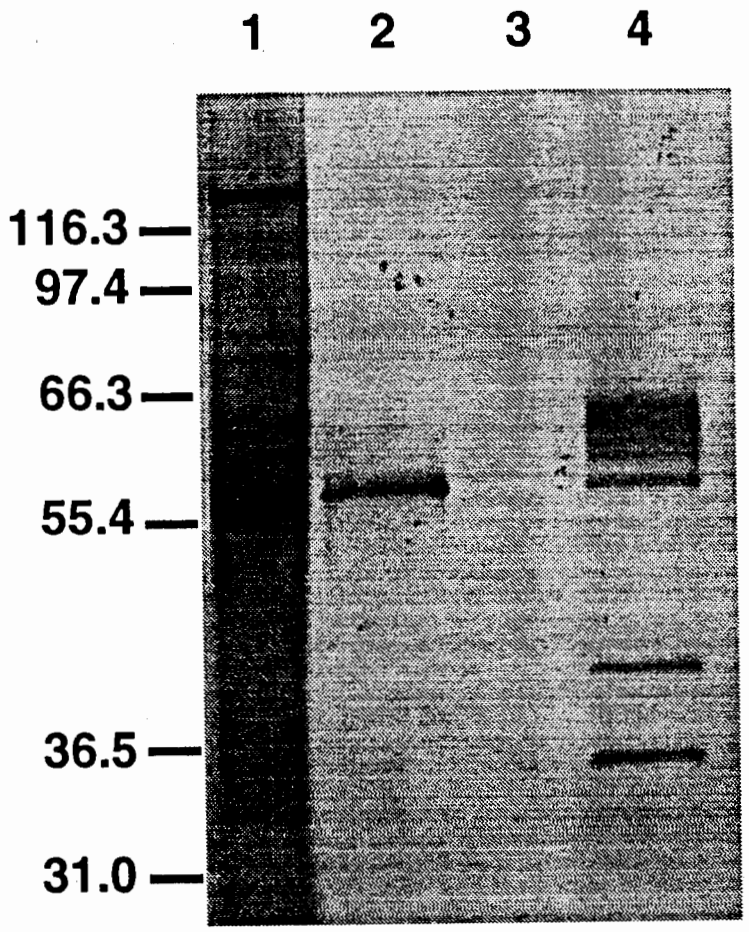

Figure 2. Immunoreactivity and molecular weight comparison of maize-expressed CryIA(b), full length native CryIA(b) and the tryptic fragment of native CryIA(b).

Samples were prepared as described in Appendixes A and C and subjected to SDS-PAGE followed by western blot analysis as described in Materials and Methods. Lane 1, 1.6 µg of full length native CryIA(b); lane 2, 0.4 µg of the tryptic fragment of native CryIA(b) (approximately 60,000 mol. wt.); lane 3, 14 µg control maize leaf protein preparation; lane 4, 10 µg CryIA(b) enriched maize leaf protein. Molecular weight markers (x 10^{-3}) are indicated.

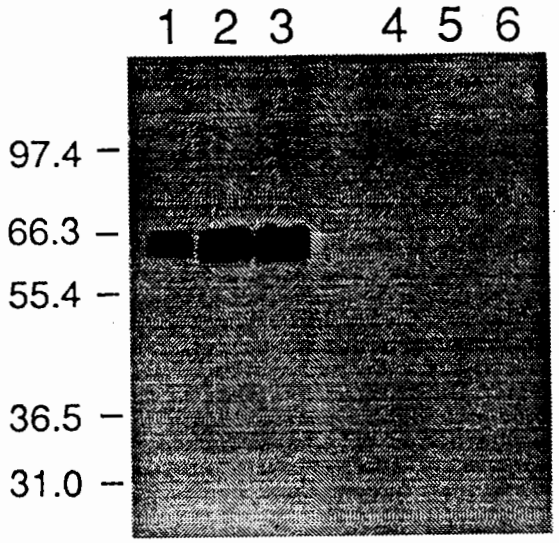

Figure 3. Western blot analysis of CryIA(b) in pollen.

Pollen extracts were prepared as described in Appendix B. Lanes 1, 2, 3 are samples containing 31, 62, and 94 µg protein, respectively, from homozygous *Bt* maize pollen extract. Lanes 4, 5, 6 are samples containing 37, 73, 110 µg protein, respectively, from nontransgenic control maize pollen extract. Molecular weight markers (x 10^{-3}) are indicated.

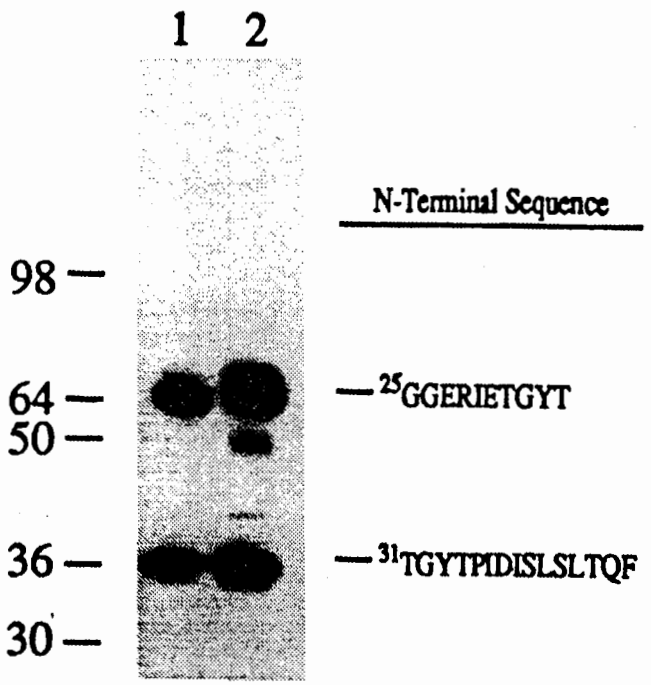

Figure 4. Western blot analysis and N-terminal sequence of CryIA(b) proteins extracted from *Bt* maize leaves.

Western blot analysis was performed as described in Materials and Methods and developed using chemiluminescence. Lane 1, 31 µg CryIA(b)-enriched maize leaf protein, containing 25 ng CryIA(b). Lane 2, 36 ng immunopurified maize expressed-CryIA(b). Molecular weight markers (x 10^{-3}) are indicated. N-terminal amino acid sequence obtained for each band is indicated.

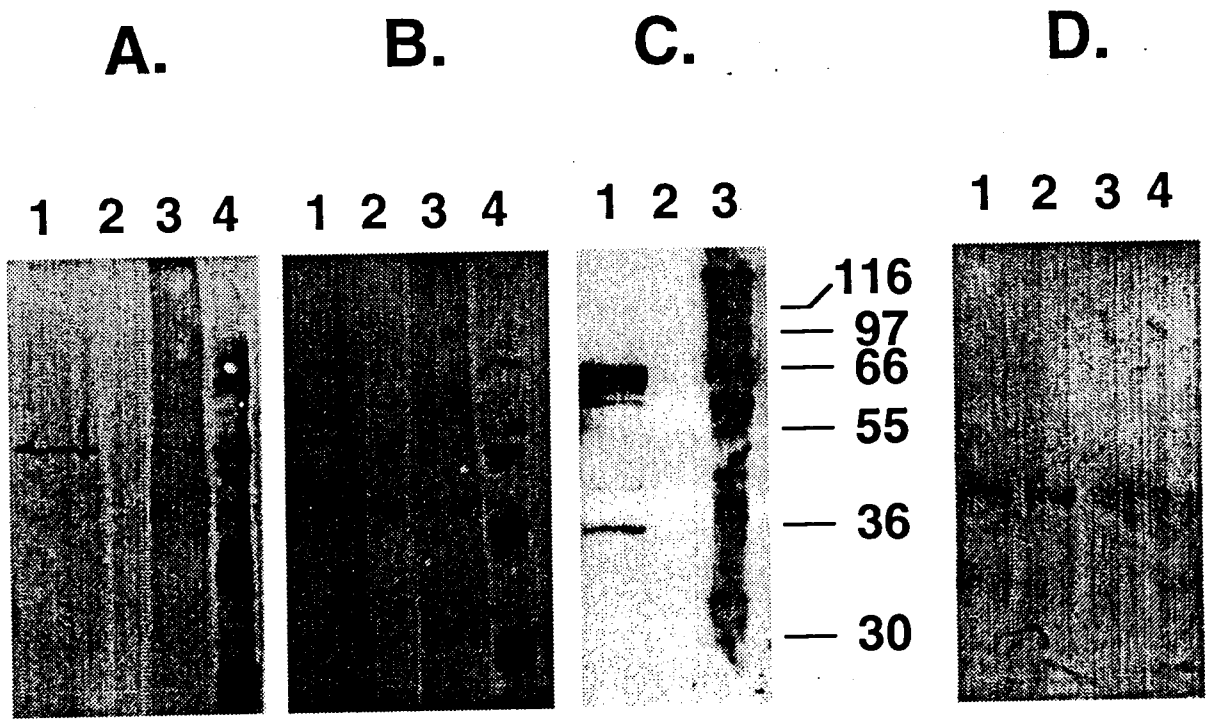

Figure 5. Analysis of post-translational glycosylation using Glycotrack® detection system.

Samples were subjected to electrophoresis on 10% polyacrylamide SDS gels, electroblotted, and developed using **A.** Glycotrack® kit without periodate treatment; **B.** Glycotrack® kit with periodate treatment; or **C.** standard western blot analysis as described in Materials and Methods using antibodies to crystal proteins from *B.t.k.* **D.** Level of sensitivity determination using Glycotrack® with periodate treatment. **A.** and **B.**, lane 1, 250 ng immunopurified maize CryIA(b); lane 2, 40 µg control maize leaf protein (negative for CryIA(b)); lane 3, 5 µg full length native CryIA(b); lane 4, biotinylated standards to monitor streptavidin binding. **C.** Lane 1, 11 ng CryIA(b)-enriched maize leaf protein; lane 2, 40 µg control maize leaf protein; lane 3, 2 µg full length native CryIA(b). **D.** Developed with periodate; lanes 1-4, 1.0, 0.5, 0.2, 0.1 µg ovalbumin, respectively. Molecular weight markers are indicated (x 10^{-3}).

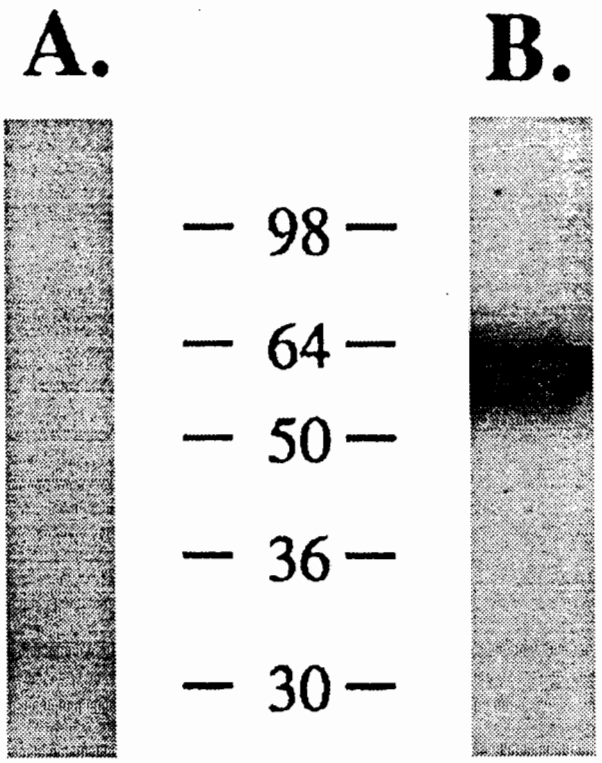

Figure 6. Attempted phosphorylation of the tryptic fragment of native CryIA(b) using maize CDPK.

A. Autoradiograph of ^{32}P-labeled samples separated by SDS-PAGE. **B.** CryIA(b) western blot analysis. Each lane contained 250 ng of the tryptic fragment of native CryIA(b) protein. Molecular weight markers are indicated (x 10^{-3}).

TABLE 1. Qualitative Comparison of Biological Activity of Maize-Expressed CryIA(b) and Native CryIA(b) from *Bacillus thuringiensis* subspecies *kurstaki* in Various Lepidopterous Species[1]

Insect	mean LC_{50}* (range) [ng CryIA(b)/cm^2 diet surface]			
	CryIA(b)-enriched maize leaf protein[2]		*B.t.k.* strain HD1-9^ cell paste	
CryIA(b)-sensitive species:				
European corn borer *Ostrinia nubilalis*	4.4	(2.8 - 5.4)	24.3	(17.5 - 30.2)
Southwestern corn borer *Diatraea grandiosella*	ND		209.5	(113.1 - 294.6)
Cabbage looper *Trichoplusia ni*	74.7	(63.5 - 85.8)	765	(528 - 1024)
Corn earworm *Helicoverpa zea*	187	(112.4 - 292.2)	978	(675 - 1320)
CryIA(b)-insensitive species:				
Fall armyworm *Spodoptera frugiperda*	> 70.8[a]		> 472.6[b]	
Black cutworm *Agrotis ipsilon*	> 70.8		> 472.6	

[1]Source: Host range comparison of native and maize expressed CryIA(b) protein (Halliday, 1994).
[2]Maize-expressed CryIA(b), ca. 65,000 mol. wt., was expected to be ca. 2-fold more potent than native CryIA(b), ca. 128,000 mol. wt., on a weight basis. Direct quantitative comparisons between maize and native CryIA(b) cannot be made because CryIA(b) could not be quantitated by the same methods in both preparations.
*LC_{50}s represent (in most cases) the mean of 3 experiments where first instar larvae were exposed to surface-treated diet for 4 days; each experiment consisted of 3 replicates of 30 larvae/replicate.
^ *B.t.k.* strain HD1-9 has been cured of plasmids such that the only Cry protein it produces is CryIA(b) (Carlton and Gonzalez, 1985; Minnich and Aronson, 1984).
ND = The test system appeared to be inappropriate for bioassaying for Southwestern corn borer as inconsistent results were obtained across the three experiments and an LC_{50} could not be determined for this test material which appeared to inhibit feeding this species.
[a]CryIA(b) LC_{90} for European corn borer, as determined by three experiments. No treatment-related mortality occurred at this dose.
[b]Intended dose was 70.8 ng CryIA(b)/cm^2, the LC_{90} for European corn borer; due to a calculation error a higher dose was applied. No treatment-related mortality was observed at this dose.

APPENDIX 3A

Isolation of Maize-Expressed CryIA(b) for Characterization Studies

INTRODUCTION

Characterization studies were performed using maize CryIA(b) present in an enriched maize leaf protein preparation. For some studies it was further purified by immunoaffinity chromatography. The extraction and partial purification protocols developed for these studies are described here.

Preparation of CryIA(b)-enriched maize leaf protein. CryIA(b) was extracted and partially purified from leaves of 3 - 6 week old greenhouse grown hybrid *Bt* maize plants (CG00615 x CG00526-176[1]) that were hemizygous for the transgenes introduced in Event 176. Leaves from negative segregants[2] of isogenic background were collected and processed in the same manner and this protein preparation was termed control maize leaf protein. Following collection the leaves were deveined, frozen in liquid nitrogen, crushed and then powdered using a mortar and pestle. Powdered leaf material was stored at -80°C until extracted. The powdered leaves were extracted by homogenization in three volumes of ice cold extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride HCl, 1 μM leupeptin, pH 10.0) per weight of powder in either a precooled CB6 Waring Industrial blender, fitted with a double blade, or with a Polytron® homogenizer (Brinkman, Westbury, NY). All processing of the extracts was conducted at 4°C or by maintaining materials chilled on ice. The extract was filtered through cheesecloth. The total volume was measured and 10% polyethylenimine was added to a final concentration of 0.15%. Extracts were centrifuged at 10,000 x g for 20 min at 4°C. The supernatant was filtered through miracloth (Calbiochem, San Diego, CA), its volume was measured, and it was brought to 40% saturation with ammonium sulfate (22.4 g/100 ml) by slow addition of crystalline ammonium sulfate with stirring at 4°C. The precipitate was collected by centrifugation at 10,000 x g for 20 min at 4°C. The pellet was resuspended in 50 mM $NaHCO_3$, 150 mM NaCl, pH 8.0 (in one tenth the original extract volume). Residual ammonium sulfate was either removed by passage over a PD-10 column (Pharmacia, Piscataway, NJ) equilibrated in extraction buffer or alternatively by dialysis against 50 mM NH_4HCO_3. The PD-10 column eluent containing CryIA(b) was used directly in characterization studies whereas the dialyzed material was lyophilized before use. This material was designated CryIA(b)-enriched maize leaf protein[3] and was estimated by ELISA to be 0.07% CryIA(b) by weight. After treatment with iodoacetamide to prevent interference by dithiothreitol present in the extraction buffer (Hill and Straka, 1988), total protein in the samples was determined by the BCA™ procedure (Pierce, Rockford, IL) with ovalbumin as the protein standard. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu,

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[1] "CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176.

[2] Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent(s). The nontransgenic genotype was confirmed by susceptibility to European corn borer.

[3] For several batches of CryIA(b)-enriched maize leaf protein, it was determined that the CryIA(b) content was approximately 30 - 40-fold higher per mg protein (as measured by ELISA) than in the initial crude leaf extract.

Columbia, MD). Further purification was achieved by immunoaffinity chromatography as described below.

Immunoaffinity purification of maize leaf CryIA(b). An immunoaffinity column was prepared by binding approximately 1 mg of immunoaffinity-purified rabbit polyclonal antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 to 0.3 ml AffiGel 15 (Bio-Rad, Hercules, CA) following the manufacturer's instructions. All manipulations of the column were conducted at 4°C. The column was equilibrated with 50 mM $NaHCO_3$, 150 mM NaCl, pH 8.0. Approximately 5 mg of CryIA(b)-enriched maize leaf protein preparation which had not been passed over a PD-10 column or dialyzed was applied to the column matrix and CryIA(b) was allowed to bind by rotating the mixture overnight at 4°C. As the column repacked by settling, unbound leaf protein was drained. The column was washed with 6 ml of 50 mM $NaHCO_3$, 150 mM NaCl, pH 8.0, followed by 3 ml of 100 mM sodium phosphate, pH 6.8, and one ml fractions were collected. Maize-expressed CryIA(b) was then eluted by sequential washes of low pH (100 mM glycine, pH 2.5), high pH (100 mM CAPS, pH 11.0) and low pH (100 mM glycine, pH 2.5) buffers as described by Harlow and Lane (1988). The pH of each fraction was adjusted by addition of 1/20th volume of 1 M sodium phosphate, pH 6.8, or 1 M sodium phosphate, pH 8.0, for the CAPS and glycine buffers, respectively.

Fractions containing CryIA(b) were identified by dot blot analysis as follows. Aliquots were applied to Immobilon-P® membrane (Millipore, Bedford, MA) under vacuum and washed twice with 10 mM Tris-HCl, 0.15 M NaCl, pH 7.5. Immunoaffinity purified polyclonal rabbit antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 were employed at a concentration of 0.133 µg antibody/ml blocking buffer (3% nonfat dry milk, 10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5) to develop the blot. Goat anti-rabbit IgG linked to horseradish peroxidase (Bio-Rad, Hercules, CA), diluted 1:3000 in blocking buffer, was used to bind to the primary antibody and was detected by chemiluminescence using an ECL™ kit (Amersham, Arlington Heights, IL). Appropriate fractions were pooled and quantitatively analyzed for CryIA(b) protein by enzyme-linked immunosorbent assays (ELISA, Tijssen, 1985) using immunoaffinity-purified polyclonal rabbit and protein G-purified goat polyclonal antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1. The lower limit of quantification of the double sandwich ELISA was approximately 3 ng CryIA(b)/ml. Absorbance at 405 nm was determined using a Tecan SLT 340 ATTC multiwell plate reader (Tecan, Research Triangle Park, NC) and results were analyzed using the Tecan SLT Soft2000 Curve fitting program (log/log.it algorithm).

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APPENDIX 3B

Extraction of CryIA(b) from *Bt* Maize Pollen

INTRODUCTION

Immunoreactivity and size of CryIA(b) produced in maize were determined using pollen extracts that were prepared as described here.

Sources of test and control pollen. Pollen was collected during the period of 26 Jul 93 - 4 Aug 93 from field-grown maize planted on 22 May 93 in Bloomington, IL. The *Bt* maize pollen was obtained from plants of inbred line CG00526-176 that were homozygous for the transgenes. The initial transformation Event 176 had been carried out in the same line, an elite inbred derived from a Lancaster-type population. The control pollen was obtained from non-transgenic plants of line CG00526. The plantings of transgenic and nontransgenic maize were established using standard agronomic practices and maintained concurrently in nearby plots subject to the same environmental conditions.

Collection, processing and storage of test and control pollen. Pollen was collected in the field and immediately cooled in an ice chest until transfer to a -20°C freezer later the same day. The samples were shipped frozen overnight on dry ice to the Ciba Agricultural Biotechnology Research Unit (Research Triangle Park, NC) where they were stored at -80°C until processing and analysis, which was initiated on 3 Feb 94. All pollen from a given genotype was pooled, filtered through 1 mm x 1 mm screening to remove anthers and aphids, and allowed to air dry overnight at room temperature. The samples were then re-filtered through a 100 µm sieve in an effort to remove the remaining small anther and aphid parts. The pollen samples were returned to -80°C storage until analyzed.

Pollen extraction and protein quantification. Extracts of transgenic and control pollen were prepared by suspending a sample 1:30 (w:v) in extraction buffer (50 mM CAPS, pH 10.0, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonylfluoride HCl, and 1 µM leupeptin). After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi, followed by centrifugation at 14,000 x g for 5 min at 4°C. After treatment with iodoacetamide to prevent interference by dithiothreitol present in the extraction buffer (Hill and Straka, 1988), total protein in the resulting extracts was determined by the BCA™ procedure (Pierce, Rockford, IL) with ovalbumin as the protein standard. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu, Columbia, MD). These extracts were used for western blot analyses.

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APPENDIX 3C

Preparation of Native CryIA(b) for Characterization Studies

INTRODUCTION

Described here is the preparation of full length native CryIA(b) δ-endotoxin from *Bacillus thuringiensis* subspecies *kurstaki* strain HD1-9, as well as the preparation of the major tryptic fragment of native CryIA(b) protein.

Source of cell paste for purification of native CryIA(b). *B.t.k.* strain HD1-9 has been cured of plasmids such that the only Cry protein it produces is CryIA(b) (Carlton and Gonzalez, 1985; Minnich and Aronson, 1984). This strain was derived from *B.t.k.* strain HD1, the source of the native *cryIA(b)* gene upon which Ciba Seeds' synthetic gene in maize was based. From 28 Apr 93 - 7 May 93, three 250-L batches of *B.t.k.* strain HD1-9[1] were prepared by the fermentation facility at the University of Minnesota and the sporulated cells were pooled and concentrated using standard techniques. The cell paste was frozen in approximately 200 g aliquots and shipped overnight on dry ice to the Ciba Agricultural Biotechnology Research Unit, Research Triangle Park, NC, where it was received on 14 May 93 and assigned the sample lot. no. HD19-UM305006. The cell paste was estimated by SDS-polyacrylamide gel electrophoresis to contain 9.5% by weight full length CryIA(b). The cell paste was stored at -20°C.

Confirmation of strain identity. To confirm the identity of the fermented strain as *B.t.k.* strain HD1-9, sample HD19-UM305006 was analyzed by the polymerase chain reaction (PCR) method (Carozzi *et al.*, 1991) using DNA primers specific for unique coding sequences in the *cryIA(a)*, *cryIA(b)*, and *cryIA(c)* genes.

Isolation of native CryIA(b) protein. Cell paste sample HD19-UM305006 was thawed in 200 g batches suspended in 1L 0.05% Triton X-100 and centrifuged at 7280 x g in a Sorvall RC3-B centrifuge (H600A rotor) for 20 min at 4°C. The pellet was washed twice in 1 L of 50 mM Tris-HCl, 0.2 M NaCl, 2 mM EDTA, pH 8.0, and centrifuged as above. The CryIA(b) crystals were solubilized in 2 L 50 mM CAPS, 25 mM β-mercaptoethanol, pH 10.0, and the spores were immediately removed by centrifugation as before. The supernatant containing solubilized CryIA(b) protein was retained, the pellet was re-extracted with the same buffer to solubilize any remaining crystals, and spores were again removed by centrifugation. Sodium acetate (3M, pH 5.2) was added to the solubilized crystals to a final concentration of 10 mM. The pH of the solution was lowered to 5.1 - 5.5 with 6 N HCl causing precipitation of CryIA(b) protein. The precipitate was removed by centrifugation at 10,000 x g for 15 min and was washed twice with 10 mM sodium acetate, pH 5.2. The resulting pellet was lyophilized overnight.

Preparation of the major tryptic fragment of native CryIA(b). Maize-expressed CryIA(b) is similar in size to the predominant tryptic fragment of native CryIA(b), which is 564 - 607 amino acids in length (depending on the trypsin used and the length of trypsin treatment) (Figure 1). To generate this fragment, 42 g of strain HD1-9 cell paste was resuspended in 200 ml 50 mM CAPS,

[1] Generated by Dr. B. C. Carlton. Strain HD1-9 is in the strain collection of the Ciba Agricultural Biotechnology Microbial Pesticides Research Program, currently maintained by Dr. Thomas Currier, Staff Scientist, Ciba Agricultural Biotechnology Research Unit.

50 mM dithiothreitol, pH 10.0, and stirred on ice for 30 min. Spores were removed by centrifugation at 16,000 x g for 10 min at 4°C followed by further centrifugation at 20,000 x g for 10 min. Trypsin (Cat. No. T360-500, Fisher Scientific, Pittsburgh, PA) was added to a final concentration of 1.2 mg/ml and the solution was stirred at room temperature for two hours. The solution was then dialyzed against 50 mM sodium acetate, pH 5.2 (Spectra/Por7, 50,000 molecular weight cutoff, Fisher Scientific, Pittsburgh, PA) overnight at 4°C. The contents of the dialysis bag were centrifuged at 10,000 x g for 10 min at 4°C and the pellet was sequentially treated by stirring on ice for 10 min in (1) 50 mM Tris-HCl, pH 7.5; (2) 50 mM Tris-HCl, pH 8.5; and (3) 50 mM CAPS, pH 10.0. Solubilized protein was separated from particulates after each treatment by centrifugation at 10,000 x g for 10 min. The supernatant of the final extraction contained approx. 2.5 mg protein /ml and was highly enriched (as determined by SDS-PAGE) for the major tryptic fragment of CryIA(b). Total protein was quantitated by the Bio-Rad Coomassie blue method (Bio-Rad, Hercules, CA) with ovalbumin as the standard.

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CHAPTER 4

Quantitative Analysis of CryIA(b) Expression in Maize Tissue

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4. Quantitative Analysis of CryIA(b) Expression in Maize Tissue

A. Summary

The range of expression of CryIA(b) protein in Ciba Seeds *Bt* maize plants derived from event 176 was determined for various plant tissues and developmental stages in three maize lines from 1993 field tests. Similar studies were performed for selected tissues of mature greenhouse-grown inbreds representing four additional genotypes as well as four backcross generations of two genotypes.

Consistent with the tissue specificity of the green tissue promoter and pollen promoter that drive *cryIA(b)* gene expression in event 176, significant levels of CryIA(b) were detected only in leaves, pollen, and whole plants. CryIA(b) was consistently below the lower limit of quantification (< 5 ng CryIA(b)/g fr. wt.; < 5 ppb) in kernels. Only trace amounts were detected in pith and roots (< 8 ng CryIA(b)/g fr. wt.; < 8 ppb).

On a per-acre basis, the highest level of CryIA(b) protein was detected at anthesis, and was estimated to be approximately 2 - 4 g/acre across the various genotypes. CryIA(b) levels were markedly lower in late-season senescing plants, for which total CryIA(b) protein/acre was estimated to be less than 0.2 g. CryIA(b) levels in leaves and pollen of anthesis stage plants appeared to be stable over four successive backcross generations of two different *Bt* maize lines, with no indication of reduced CryIA(b) expression. Generally, CryIA(b) levels for any given tissue and growth stage varied by less than 10-fold between individual plants and between genotypes when grown under similar environmental conditions. Among all plants analyzed, the highest CryIA(b) levels detected in any individual sample of leaf, pollen or whole plants were approximately 4.4 µg/g fr. wt. (4.4 ppm), 7.1 µg/g fr. wt. (7.1 ppm), and 0.6 µg/g fr. wt. (0.6 ppm), respectively. The highest mean CryIA(b) level as a proportion of total plant protein, 14.4 µg/g total protein, was observed in a homozygous *Bt* inbred line at anthesis.

B. Introduction

To assess the range of expression of the transgenic proteins in *Bt* maize plants derived from event 176, the following analyses were conducted by enzyme-linked immunosorbent assays (ELISA):

(1) CryIA(b) level was determined for various plant tissues and developmental stages in three maize lines from 1993 field tests. Levels of CryIA(b) present on a per-acre basis were also estimated for four stages of plant development for the *Bt* maize lines evaluated.

(2) stability of CryIA(b) expression over four successive backcross generations was examined in leaves and pollen of greenhouse grown plants representing two inbred maize lines;

(3) CryIA(b) level was determined for selected tissues of four different greenhouse-grown *Bt* inbred maize lines;

Extraction efficiency experiments were performed to estimate the relative amount of CryIA(b) that is extracted during routine procedures compared with that which remains associated with the insoluble plant tissue pellet. Additionally, spike and recovery experiments were conducted to estimate the proportion of a purified CryIA(b) spike recoverable from tissue extracts prepared according to standard methods.

C. Materials and Methods

CryIA(b) Protein Quantification. The ELISA protocol for quantification of CryIA(b) protein in maize tissues and whole plants is detailed in Appendix 4A. CryIA(b) extraction efficiency and spike and recovery experiments are described in Appendices 4B and 4C. CryIA(b) concentrations given in this report are not corrected for efficiency of extraction or recovery unless specifically stated.

Source of Plants for Developmental Study. CryIA(b) levels were determined for various plant tissues and developmental stages in three maize lines from 1993 field tests. Except for seedlings, which were greenhouse grown at the Ciba Agricultural Biotechnology Research Unit (Research Triangle Park, NC), all plants evaluated in this part of the study were field grown. Planting occurred on May 22, 1993, in Bloomington, Illinois, using standard agronomic practices. Transgenic plants from an inbred line, CG00526-176,[1] and two hybrid crosses, CG00554 x CG00526-176 and CG00664 x CG00526-176, were analyzed. Transgenic plants and the corresponding isogenic, nontransgenic controls were maintained concurrently in nearby plots subject to the same environmental conditions. Plants were harvested at four developmental stages:

(1) seedling, 5 - 6 leaf stage, three weeks after planting;

(2) anthesis (pollen shed), 10 - 11 weeks after planting;

(3) seed maturity, 18 - 20 weeks after planting; and

(4) senescence, 23 weeks after planting.

At each stage the entire plant with roots was harvested intact, bagged and labeled, and shipped overnight on wet ice to the Ciba Agricultural Biotechnology Research Unit. Upon receipt, several plants were separated into their respective tissues; the remainder were kept intact for later determination of whole plant levels of CryIA(b) protein. Total weight and leaf number were recorded for each plant. Plants were then stored at -80°C until processed and extracted (see below; **Plant processing** and **Tissue extraction**). The various tissue samples and whole plants were quantitatively analyzed for CryIA(b) by ELISA. In separate handling experiments using locally field-grown *Bt* maize plants, no significant differences in CryIA(b) levels were found in leaves of freshly-harvested plants versus leaves of plants that had been subjected to comparable shipping and storage conditions as had prevailed in transporting the plants from Bloomington, IL to Research Triangle Park, NC (data not shown).

Source of Plants for Four Generation Study. The stability of CryIA(b) protein expression in leaves and pollen was evaluated in plants representing successive generations in the breeding process. Seed was collected from four successive backcross[2] generations (BC1, BC2, BC3, and BC4) derived by using line CG00526-176 as the source of the transgenes and either CG00554 or CG00642 as the recurrent parent. These seed were planted concurrently in the greenhouse and the seedlings were bioassayed

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[1]"CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176. CG00526-176 is an inbred line that is homozygous for the transgenes.

[2]Inbred conversion to introgress the *cryIA(b)* gene from one inbred line into a second required successive backcrosses using the second inbred as the recurrent parent.

against European corn borer (ECB), the target pest, to cull out negative segregants.[3] The remaining plants were grown in the greenhouse to anthesis at which time the uppermost leaf and the tassel were removed from each plant for analysis. Leaf and pollen samples were extracted as described below in **Plant Processing** and **Tissue Extraction** and quantitatively analyzed for CryIA(b) by ELISA.

Plant Source for Inbred Comparisons. The level of CryIA(b) was evaluated in selected tissues of four *Bt* maize inbred lines. The transgene had been introgressed into these lines from the initial homozygous inbred, CG00526-176, by three successive backcrosses. Following self-fertilization, individual plants were identified as homozygous for the transgenes by bioassaying progeny of test crosses with nontransgenic plants against ECB and observing uniform insecticidal activity.[4] Mature leaves of confirmed homozygous, greenhouse-grown plants from lines CG00554-176, CG00615-176, CG00642-176, and CG00716-176 were harvested 13 weeks after planting and analyzed for CryIA(b) levels. Kernels were harvested from all four lines at seed maturity and also analyzed for CryIA(b) protein. CryIA(b) levels were additionally determined for the two lines for which pollen samples were available. Tissue extracts were prepared as described below (see **Plant processing** and **Tissue extraction**) and analyzed for CryIA(b) levels by ELISA.

Plant Source for Estimations of Grams of CryIA(b) Protein Per Acre. CryIA(b) protein values obtained for whole plants from the three lines analyzed for the developmental study (see above; **Plant source for developmental study**) were used to estimate the amount of CryIA(b) protein that may be produced per acre of *Bt* maize. The following calculations were made assuming 25,000 plants per acre:

$$\text{g CryIA(b)/acre} = \frac{\text{g CryIA(b)}}{\text{g fr. wt.}} \times \frac{\text{g fr. wt.}}{\text{plant}} \times \frac{\text{25,000 plants}}{\text{acre}}$$

Plant Processing. For all analyses of CryIA(b) levels in whole plants, frozen tissue was reduced to a fine powder by grinding in an ice grinder followed by further treatment in a coffee grinder. The ground material was well mixed to ensure homogeneous sampling. Assessment of CryIA(b) protein in leaves was accomplished by crushing the entire complement of leaves from a single plant in liquid nitrogen and mixing thoroughly. An aliquot was then ground further to a fine powder using a mortar and pestle. For all analyses of kernels, roots and pith, the entire frozen sample was ground to a fine homogeneous powder using a coffee grinder. During processing, all samples were maintained in the frozen state by liquid nitrogen or dry ice. An aliquot was removed for lyophilization for dry weight determination. Samples for ELISA analysis were stored at -80°C until extracted. To estimate total protein, the percent elemental nitrogen was determined on selected lyophilized samples of whole plants and kernels by Southern Testing and Research Laboratories, Inc., Wilson, NC following AOAC Method 990.03.

Pollen was collected from individual tassels by homogenizing spikelets in a Waring blender (using two four-second pulses) in 50 ml extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl)

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[3]Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent(s).

[4]A cross of a homozygous *Bt+/Bt+* parent with a *Bt-/Bt-* (wild-type) parent yields only one progeny genotype, *Bt+/Bt-*. Because the *Bt* trait behaves as a dominant gene all progeny are thus insecticidal when challenged with ECB. In contrast, a cross of a hemizygous *Bt+/Bt-* parent with a *Bt-/Bt-* parent would produce 50% *Bt+/Bt-* (ECB active) and 50% *Bt-/Bt-* progeny.

benzenesulfonylfluoride HCl, 1 mM leupeptin, pH 10.0). Pollen was separated from anther and tassel material by filtration through cheesecloth into parachute cloth. The pollen was washed off the parachute cloth into a centrifuge tube, the buffer removed by aspiration after the pollen had settled, and the pollen was lyophilized.

Tissue Extraction. All tissues, except for pollen, were extracted as follows. Approximately 3 g of the powdered tissue was extracted in 2 - 3 volumes of extraction buffer using a Polytron® homogenizer (Brinkman, Westbury, NY). The homogenized material was filtered through cheesecloth which had been prewetted with extraction buffer. The filtrate was centrifuged at 10,000 x g for 15 min. The supernatant was retained and its volume was measured. This extract was used for CryIA(b) analysis by ELISA. After treatment with iodoacetamide as described by Hill and Straka (1988), total protein in the extracts was quantitated using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL). Ovalbumin was used as the protein standard. Three different volumes of each sample were assayed to obtain at least two absorbance readings that coincided with the standard curve. The mean of these readings was used to calculate total protein. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

D. Results

CryIA(b) Protein Levels During Maize Development. CryIA(b) protein was detected in significant quantities only in leaves and pollen, as would be expected based on the tissue specificities of the gene promoters which drive *cryIA(b)* gene expression in event 176. Whole plant CryIA(b) levels per g of fresh or dry weight were highest at seedling stage, with levels decreasing during the growing season (Tables 1 and 2). Kernels, roots and pith had nonquantifiable trace levels. Leaf, pollen and whole plant samples from homozygous and hemizygous plants had overlapping ranges of measured CryIA(b) levels. Although the homozygous *Bt* inbred plants could theoretically have been expected to express higher concentrations of CryIA(b) protein than the hemizygous hybrids, no single genotype appeared to consistently express higher levels per g fresh or dry weight at all sampling times for all tissues.

Within a given genotype, variability in CryIA(b) levels among individual plants was always less than 10-fold (i.e., within the same order of magnitude); this was true for determinations made on both a fresh weight and dry weight basis for all tissues and whole plants at any given time point where CryIA(b) was quantifiable. The variability in CryIA(b) levels between genotypes was also less than 10-fold, except for whole plants at anthesis where the range across all individual plants for all genotypes was 14 - 213 ng CryIA(b)/g fr. wt., corresponding to 0.05 - 1.94 µg CryIA(b)/g dry wt.

Selected whole plant and kernel samples were analyzed for total protein per g dry weight by determining the percent elemental nitrogen for lyophilized samples. Values for total protein per g dry wt. were used to calculate the quantity of CryIA(b) detected as a proportion of the total plant protein (Table 3). At anthesis, the stage at which plants reach their approximate maximum vegetative biomass, mean CryIA(b) values per g of total plant protein ranged from 2.5 - 14.4 µg across the three *Bt* maize genotypes. Whole plants at seed maturity had mean values of 2.1 - 3.7 µg CryIA(b)/g total plant protein. There were no substantial differences in percent nitrogen in transgenic vs. nontransgenic whole plants or kernels of isogenic backgrounds.

CryIA(b) Protein Levels in Four Successive Generations of *Bt* Maize Plants. Leaves and pollen of anthesis stage plants from all four backcross generations during inbred conversion of two *Bt* maize lines were analyzed for CryIA(b) levels. CryIA(b) protein

levels in both leaves and pollen appeared to be stable with no indication of reduced CryIA(b) expression over these four generations (Table 4).

CryIA(b) Protein Levels in Additional Homozygous Inbred *Bt* Maize Lines. Although relatively few plants were available for analysis, variability in CryIA(b) protein levels among mature leaves of four greenhouse-grown homozygous transgenic inbred lines was less than four-fold (0.25 - 0.96 µg CryIA(b) protein/g fr. wt., corresponding to 0.74 - 2.66 µg CryIA(b)/g dry wt.) (Table 5). CryIA(b) protein was present at trace levels in kernels of all four inbred lines but was consistently below the lower limit of quantification. CryIA(b) protein levels in pollen were comparable in the two lines examined.

Total CryIA(b) Per Acre. Estimates of total CryIA(b) protein per acre did not appear to vary significantly between the three maize lines at each developmental stage (Table 6); peak levels occurred at anthesis (1.59 - 4.15 g CryIA(b)/acre). Since only traces levels of CryIA(b) were detected in whole senescing plants, estimates of total CryIA(b)/acre were calculated assuming CryIA(b) was present at the lower limit of CryIA(b) quantification for this tissue, 8 ng/g fr. wt. The values obtained by such calculations, 0.08 - 0.19 g CryIA(b)/acre, therefore represent an upper-bound estimate and were approximately 5% of the peak levels found in whole plants at anthesis. Total CryIA(b) values per individual plant were all within an order of magnitude for any given developmental stage, both within and between genotypes.

Extraction Efficiency Experiments. Extraction efficiency of CryIA(b) was determined to be approximately 87% from leaves, 85% from pollen and 75% from whole plants (Appendix 4B).

Spike and Recovery Experiments. Spike and recovery experiments indicated that recovery of a purified CryIA(b) spike from tissue extracts was approximately 31% for leaves, 64% for kernels, 73% for whole plants and 100% for pollen (Appendix 4C).

TABLE 1. CryIA(b) Protein Levels on a Fresh Weight Basis During *Bt* Maize Development, Summer 1993

Tissue	Genotype, Maize Line		mean ng CryIA(b)/g fr. wt. (N; range) Seedling[1]	Anthesis	Seed Maturity	Senescence
Leaves	+/+	CG00526-176	1159 (3; 892-1506)	735 (3; 657-793)	465 (3; 158-922)	66 (2; 55-77)
	+/-	CG00554 x CG00526-176	596 (5; 308-738)	530 (3; 449-614)	471 (3; 266-765)	88 (3; 57-141)
	+/-	CG00564 x CG00526-176	839 (3; 285-1253)	3029 (2; 1631-4427)	442 (2; 365-520)	225 (3; 47-379)
Roots	+/+	CG00526-176	< 8 (2)	< 8 (4)	< 8 (4)	na
	+/-	CG00554 x CG00526-176	< 8 (6)	< 8 (3)	< 8 (3)	na
	+/-	CG00564 x CG00526-176	< 8 (1)	< 8 (3)	< 8 (2)	na
Pith	+/+	CG00526-176	na	< 8 (4)	< 8 (4)	na
	+/-	CG00554 x CG00526-176	na	< 8 (3)	< 8 (3)	na
	+/-	CG00564 x CG00526-176	na	< 8 (3)	< 8 (2)	na
Pollen*	+/+	CG00526-176	---	2021 (4; 1732-2611)	---	---
	+/-	CG00554 x CG00526-176	---	1137 (3; 828-1474)	---	---
	+/-	CG00564 x CG00526-176	---	2348 (3; 2228-2438)	---	---
Kernels	+/+	CG00526-176	---	---	< 5 (4)	< 5 (5)
	+/-	CG00554 x CG00526-176	---	---	< 5 (3)	< 5 (3)
	+/-	CG00564 x CG00526-176	---	---	< 5 (2)	< 5 (3)
Whole Plant	+/+	CG00526-176	315 (3; 191-532)	182 (5; 159-213)	73 (4; 50-107)	< 8 (5)
	+/-	CG00554 x CG00526-176	230 (6; 81-556)	44 (3; 14-88)	41 (4; 21-68)	< 8 (4)
	+/-	CG00564 x CG00526-176	316 (2; 305-328)	144 (3; 102-167)	71 (4; 48-92)	< 8 (3)

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng CryIA(b)/g fr. wt. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue. Plants that were homozygous or hemizygous for the transgenes were designated "+/+" or "+/-", respectively. --- = not applicable (tissue not available at this developmental stage); na = not analyzed.

[1]Seedlings were greenhouse grown and analyzed three weeks after planting; all other stages were field-grown.

*Values were determined on dried pollen samples and extrapolated to fresh wt. by multiplying µg CryIA(b)/g dry wt. pollen by 0.468 (g dry wt. pollen/g fr. wt. pollen).

TABLE 2. CryIA(b) Protein Levels on a Dry Weight Basis During *Bt* Maize Development, Summer 1993

Tissue	Genotype, Maize Line		mean µg CryIA(b)/g dry wt. (N; range) Seedling[1]	Anthesis	Seed Maturity	Senescence
Leaves	+/+	CG00526-176	10.50 (3; 8.57-13.09)	3.04 (3; 2.84-3.43)	1.43 (3; 0.46-2.70)	0.10 (2; 0.09-0.10)
	+/-	CG00554 x CG00526-176	4.78 (5; 2.41-5.95)	2.70 (3; 2.22-3.36)	1.65 (3; 0.95-2.90)	0.12 (3; 0.08-0.19)
	+/-	CG00564 x CG00526-176	7.56 (3; 2.56-11.28)	13.37 (2; 7.20-19.54)	1.52 (2; 1.25-1.78)	0.30 (3; 0.06-0.50)
Roots	+/+	CG00526-176	< 0.10 (2)	< 0.04 (4)	< 0.04 (4)	na
	+/-	CG00554 x CG00526-176	< 0.10 (6)	< 0.04 (3)	< 0.04 (3)	na
	+/-	CG00564 x CG00526-176	< 0.10 (1)	< 0.04 (3)	< 0.04 (2)	na
Pith	+/+	CG00526-176	na	< 0.07 (4)	< 0.04 (4)	na
	+/-	CG00554 x CG00526-176	na	< 0.07 (3)	< 0.04 (3)	na
	+/-	CG00564 x CG00526-176	na	< 0.07 (3)	< 0.04 (2)	na
Pollen	+/+	CG00526-176	---	4.32 (4; 3.70-5.58)	---	---
	+/-	CG00554 x CG00526-176	---	2.34 (3; 1.77-3.15)	---	---
	+/-	CG00564 x CG00526-176	---	5.01 (3; 4.76-5.21)	---	---
Kernels	+/+	CG00526-176	---	---	< 0.01 (4)	< 0.01 (5)
	+/-	CG00554 x CG00526-176	---	---	< 0.01 (3)	< 0.01 (3)
	+/-	CG00564 x CG00526-176	---	---	< 0.01 (2)	< 0.01 (3)
Whole Plant	+/+	CG00526-176	4.19 (3; 2.45-7.45)	1.44 (5; 0.93-1.94)	0.29 (4; 0.10-0.49)	< 0.02 (5)
	+/-	CG00554 x CG00526-176	2.85 (6; 0.75-7.33)	0.20 (3; 0.05-0.44)	0.15 (4; 0.09-0.25)	< 0.02 (4)
	+/-	CG00564 x CG00526-176	3.40 (2; 3.27-3.52)	0.74 (3; 0.52-0.86)	0.26 (4; 0.17-0.33)	< 0.02 (3)

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng CryIA(b)/g fr. wt. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue. Plants that were homozygous or hemizygous for the transgenes were designated "+/+" or "+/-", respectively. --- = not applicable (tissue not available at this developmental stage); na = not analyzed.

[1]Seedlings were greenhouse grown and analyzed three weeks after planting; all other stages were field-grown.

TABLE 3. CryIA(b) Levels in Field-Grown Whole Plants and Kernels on a Total Protein Basis

Sample Stage	Genotype, Maize Line		mean % N[1]	(N; range)	g protein[2] / g dry wt.	µg CryIA(b)[3] / g protein
Whole Plants at Anthesis	-/-	CG00526[a]	1.61	(3; 1.36-1.78)	0.10	0
	+/+	CG00526-176	1.62	(5; 1.30-1.80)	0.10	14.40
	-/-	CG00554xCG00526[a]	1.49	(3; 1.28-1.72)	0.09	0
	+/-	CG00554xCG00526-176	1.20	(3; 1.04-1.46)	0.08	2.50
	-/-	CG00564xCG00526[a]	1.47	(2; 1.34-1.60)	0.09	0
	+/-	CG00564xCG00526-176	1.52	(3; 1.39-1.63)	0.10	7.40
Whole Plants at Seed Maturity	-/-	CG00526[a]	1.40	(3; 1.28-1.48)	0.09	0
	+/+	CG00526-176	1.27	(4; 1.15-1.34)	0.08	3.63
	-/-	CG00554xCG00526[a]	1.19	(3; 0.88-1.57)	0.07	0
	+/-	CG00554xCG00526-176	1.13	(4; 0.83-1.34)	0.07	2.14
	-/-	CG00564xCG00526[a]	1.16	(2; 0.80-1.52)	0.07	0
	+/-	CG00564xCG00526-176	1.10	(4; 1.08-1.13)	0.07	3.71
Kernels at Seed Maturity	-/-	CG00526[a]	1.74	(3; 1.59-1.83)	0.11	0
	+/+	CG00526-176	1.80	(4; 1.58-2.02)	0.11	< 0.09
	-/-	CG00554xCG00526[a]	1.82	(3; 1.72-2.01)	0.11	0
	+/-	CG00554xCG00526-176	1.83	(3; 1.69-1.91)	0.11	< 0.09
	-/-	CG00564xCG00526[a]	1.47	(2; 1.46-1.47)	0.09	0
	+/-	CG00564xCG00526-176	1.65	(2; 1.46-1.84)	0.10	< 0.10

[a]Values for wild type and negative segregants were pooled. Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent(s).

[1]Elemental nitrogen (% N on a dry weight basis)

[2]Total protein per dry wt. tissue = mean % N x 6.25/100 (Conversion factor for maize, Watt and Merrill, USDA Handbook 8 - Food Composition, 1975).

[3] µg CryIA(b)/g protein = $\frac{\text{µg CryIA(b)}}{\text{g dry wt.}}$ x [1/(g protein/g dry wt.)]

Mean CryIA(b) values obtained from Table 2 were used in these calculations.

"-/-" = nontransgenic; "+/-" = hemizygous for transgenes in Event 176; "+/+" = homozygous for transgenes in Event 176.

TABLE 4: CrylA(b) Levels In Several Backcross Generations During *Bt* Introgression into Inbred Lines CG00642 and CG00554

Generation	N	mean CrylA(b) levels (range) Leaves (at Anthesis) µg/g fr. wt.	Leaves (at Anthesis) µg/g dry wt.	Pollen µg/g fr. wt.*	Pollen µg/g dry wt.
CG00642 Recurrent Parent					
BC1[a]	8	1.33 (0.78-2.31)	6.42 (4.36-9.24)	1.61 (1.36-1.88)	3.43 (2.90-4.01)
BC2	3	2.49 (2.13-3.00)	10.24 (8.52-11.11)	1.99 (1.64-2.18)	4.25 (3.51-4.66)
BC3	4	2.08 (1.45-2.92)	8.89 (7.25-12.18)	2.09 (1.53-3.04)	4.47 (3.27-6.49)
BC4	3	1.86 (1.59-2.05)	7.77 (6.61-8.83)	2.08 (1.83-2.58)	4.45 (3.90-5.51)
CG00554 Recurrent Parent					
BC1	2	1.12 (0.85-1.39)	5.13 (4.70-5.56)	1.79 (1.78-1.80)	3.82 (3.81-3.84)
BC2	5	1.68 (0.90-2.58)	7.81 (4.50-9.10)	2.74 (2.05-3.29)	5.86 (4.38-7.02)
BC3	2	1.23 (0.73-1.73)	6.58 (4.06-10.30)	3.13 (2.56-3.70)	6.68 (5.46-7.90)
BC4	5	2.22 (1.37-3.15)	11.08 (7.00-15.76)	2.45 (2.22-2.63)	5.23 (4.74-5.63)

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng CrylA(b)/g fr. wt.
*Values obtained by multiplying µg CrylA(b)/g dry wt. by 0.468 (g dry wt./g fr. wt. for pollen).
[a]Backcross generation

TABLE 5. CrylA(b) Levels in Several Greenhouse Grown Homozygous *Bt* Maize Inbred Lines

Tissue/Stage	Inbred Line	N[1]	mean CrylA(b) level (range) µg/g fr. wt.	µg/g dry wt.
Leaves at Seed Maturity	CG00554-176	1	0.25	0.74
	CG00615-176	1	0.96	2.46
	CG00642-176	3	0.66 (0.51-0.80)	2.10 (1.19-2.66)
	CG00716-176	5	0.52 (0.34-0.82)	1.79 (1.07-2.50)
Kernels at Seed Maturity	CG00554-176	1	< 0.01	< 0.01
	CG00615-176	1	< 0.01	< 0.01
	CG00642-176	3	< 0.01	< 0.01
	CG00716-176	5	< 0.01	< 0.01
Pollen	CG00554-176	2	5.71* (4.35-7.07)	12.20 (9.30-15.11)
	CG00642-176	1	4.08*	8.72

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng CrylA(b)/g fr. wt. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue.

*Values were determined on dried pollen samples and extrapolated to fresh wt. by multiplying µg CrylA(b)/g dry wt. pollen by 0.468 (g dry wt. pollen/g fr. wt. pollen).

[1]Total number of plants available for analysis

TABLE 6. Estimated CrylA(b) Levels per Acre of *Bt* Maize During the Growing Season

Stage	Geno-type	Maize Line	N	mean mg CrylA(b) plant (range)	g CrylA(b)* acre
Seedling	+/+	CG00526-176	2	6.94 (5.07-8.81)	0.17
	+/-	CG00554 x CG00526-176	3	10.96 (8.26-14.11)	0.27
	+/-	CG00564 x CG00526-176	2	9.39 (8.49-10.29)	0.23
Anthesis	+/+	CG00526-176	5	138.39 (113.41-170.44)	3.46
	+/-	CG00554 x CG00526-176	3	63.58 (20.81-132.37)	1.59
	+/-	CG00564 x CG00526-176	3	166.04 (119.49-190.77)	4.15
Seed Maturity	+/+	CG00526-176	4	66.77 (44.36-100.39)	1.67
	+/-	CG00554 x CG00526-176	4	65.5 (30.96-95.33)	1.64
	+/-	CG00564 x CG00526-176	4	107.23 (58.97-149.50)	2.68
Senescence^	+/+	CG00526-176	5	< 3.23 (< 2.80-< 3.53)	< 0.08
	+/-	CG00554 x CG00526-176	4	< 6.12 (< 5.56-< 7.04)	< 0.15
	+/-	CG00564 x CG00526-176	3	< 7.68 (< 7.64-< 7.74)	< 0.19

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. Plants that were homozygous or hemizygous for the transgenes were designated "+/+" or "+/-", respectively.

*25,000 plants per acre used in this calculation

^Since only trace levels of CrylA(b) were detected in senescing plants, the lower limit of quantification for this tissue, 8 ng CrylA(b)/g fr. wt., was used in these calculations. The estimated values for g CrylA(b) per acre are therefore indicated as less than ("<") the derived value.

APPENDIX 4A

CryIA(b) ELISA Protocol

Introduction

Extracts were quantitatively analyzed for CryIA(b) protein by enzyme-linked immunosorbent assays (ELISA) (Tijssen, 1985) using immunoaffinity-purified polyclonal rabbit and protein G-purified polyclonal goat antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1. The ELISA method described here was adapted from procedures initially optimized for leaf tissue. The protocol, conditions employed, and sample calculations are described in detail.

Materials and Methods

ELISA Buffers.

Borate Buffered saline (BBS)

100 mM Boric Acid
25 mM Sodium Borate
75 mM NaCl
pH 8.4 - 8.5

Blocking Buffer

10 mM Sodium Phosphate pH 7.4
140 mM NaCl
1% Bovine Serum Albumin (BSA)
0.02% Sodium azide

Diluent

10 mM Sodium Phosphate, pH 7.4
140 mM NaCl
0.05% Tween-20
1% BSA
0.02% Sodium Azide

Wash Buffer

10 mM Tris-HCl, pH 8.0
0.05% Tween-20
0.02% Sodium Azide

Phosphatase Substrate Buffer

10 mM Diethanolamine, pH 9.8
5 mM $MgCl_2$

CryIA(b) ELISA Protocol.

Vinyl 96-well plates (Costar, Cambridge, MA) were pre-washed for two hours with 95% ethanol and dried overnight. The plates were then coated with immunopurified polyclonal rabbit antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 (1 mg/ml in borate buffered saline) and incubated overnight at 4°C. Plates were washed three times with wash buffer and blocked for 45 min at room temperature with blocking buffer. Plates were washed three times and triplicate samples of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume was 50 ml). Following incubation at 4°C for 2 hr the plates were washed three times and coated with protein-G purified polyclonal goat antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 (0.75 mg/ml diluent). Plates were incubated for 1.5 h at 37°C and then washed three times prior to coating with rabbit anti-goat alkaline phosphatase conjugated antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted to 2 mg/ml in diluent. Plates were incubated at 37°C for 1 hour, washed three times and

phosphatase substrate (0.6 mg p-nitrophenyl phosphate/ml phosphatase substrate buffer) was added. Color was allowed to develop for 30 min and the reactions were stopped by addition of 3 N NaOH. Absorbance at 405 nm was determined using a Tecan SLT 340 ATTC multiwell plate reader (Tecan, Research Triangle Park, NC) and results were analyzed using the Tecan SLT Soft2000 Curve fitting program (log/log.it algorithm).

Temperatures and incubation times were critical as the ELISA was not designed to go to equilibrium. Standard curves, prepared using purified trypsin-treated native CryIA(b) diluted in diluent, were run daily and usually on each plate. Only the linear range of the standard curve, between 3 and 20 ng CryIA(b), was used for quantitation. CryIA(b) was considered to be nondetectable if the mean absorbance obtained for the triplicate ELISA samples did not exceed that of a wild type control or a negative segregant. Inclusion of extracts from tissues of nontransgenic plants in the standards had essentially no effect on the linearity range or the mean absorbance of standards. This demonstrated that there was essentially no background caused by nonspecific binding to plant proteins. The lower limit of quantification, below which CryIA(b) may be detectable but not quantifiable, was estimated for various plant tissues at different developmental stages. On a fresh weight basis there was little difference in this value between developmental stages for a given plant tissue. The most variable tissue was leaves, which had lower limits of quantification ranging from 5 - 9 ng CryIA(b) per g fr. wt. The 9 ng/g fr. wt. value was designated as the lower limit of quantification and is shown in Table A-1. On a dry weight basis, however, different lower limits of quantification were applicable to most tissues at different developmental stages, due to varying moisture content of the tissues.

TABLE 4A-1: Approximate Lower Limits of Quantification of CryIA(b) in *Bt* Maize Tissues Derived from Event 176

Tissue	ng CryIA(b) g fr. wt.	mg CryIA(b) g dry wt.			
	All stages analyzed	Seedling	Anthesis	Seed Maturity	Senescence
Leaves	9	0.01	0.03	0.02	0.01
Kernels	5	*	*	0.01	0.01
Pith	8	*	0.07	0.04	*
Roots	8	0.10	0.04	0.04	*
Pollen	44	*	0.09	*	*
Whole Plants	8	0.09	0.05	0.02	0.02

*Tissue was not analyzed at this stage.

$$\text{Lower limit of quantification} = \frac{\text{lower limit of standard curve}}{\text{g fr. wt./ml extract at max. conc. usable in ELISA}}$$

Sample Calculation:

$$\text{For kernels: } \frac{3 \text{ ng CryIA(b)/ml}}{0.63 \text{ g fr. wt./ml extract}} = 4.7 \text{ ng CryIA(b)/g fr. wt}$$

(Value was rounded up to 5 ng CryIA(b)/g fr. wt. for Table 1).

CryIA(b) was determined to be stable for at least 5 days at 4∞C in extracts prepared as described in Tissue extraction, in the main text of this report. Range finder ELISA assays were performed to allow dilutions of extracts to be prepared such that mean absorbance of three replicates closely

coincided with the midpoint of the linear portion of the standard curve. Only analyses in which the coefficients of variance were less than 10% were accepted.

Processing and extractions were as described in **Plant processing** and **Tissue extraction** sections of main text in this volume. Generally, 2 - 5 plants were sampled for each time point, with one extract prepared from each sample. Extracts of different samples of the same tissue specimen yielded consistent data (Table 4A-2).

TABLE 4A-2: CryIA(b) Determinations on Extracts of Different Samples of the Same Tissue Specimen

Tissue		ng CryIA(b)/g fr. wt.	Mean ± S.D.
Whole Plant at Seed Maturity		81.8 93.8 118.6	98.1 ± 15.3
Leaves at Seed Maturity		993.6 1070.1	1031.9 ± 38.3
Whole Plant at Anthesis	Exp. 1	210.5 158.3 128.3	165.7 ± 34.0
	Exp. 2	149.7 189.7 139.3 196.9	168.9 ± 24.8

CryIA(b) calculations:

$$\frac{\text{ng CryIA(b)}}{\text{ml extract}} = \frac{\text{ng CryIA(b)}^{1}}{\text{ml}} \times \frac{\text{50 ml/well}}{\text{ml extract/well}}$$

$$\frac{\text{ng CryIA(b)}}{\text{g fr. wt.}} = \frac{\text{ng CryIA(b)}}{\text{ml extract}} \times \frac{\text{ml extract}}{\text{g fr. wt.}}$$

$$\%\ \text{dry weight} = \frac{\text{dry wt.}}{\text{fr. wt.}} \times 100$$

$$\frac{\text{ng CryIA(b)}}{\text{g dry wt.}} = \frac{\text{ng CryIA(b)}}{\text{g fr. wt.}} \times \frac{\text{g fr. wt.}}{\text{g dry wt.}}$$

REFERENCES

Tijssen, P. (1985) Processing of data and reporting of results of enzyme immunoassays. In, *Practice and theory of enzyme immunoassays.* (Laboratory techniques in biochemistry and molecular biology, V. 15) Elsevier Science Publishers, Amsterdam, The Netherlands, pp. 385-421.

[1]ELISA value

APPENDIX 4B

Extraction Efficiency of CryIA(b)

INTRODUCTION

Extraction efficiency measurements were performed to estimate the relative amount of CryIA(b) that is extracted during routine procedures compared with that which remains associated with the insoluble plant tissue pellet. Tissues examined were leaf, pollen, and whole plant. Other tissues contained levels of CryIA(b) too low for meaningful data to be obtained in this kind of experiment.

MATERIALS and METHODS

Source of Plant Material. Leaves from mature field grown, hemizygous CG00554 x CG00526-176[2] hybrid plants were used in these studies. Pollen was obtained from tassels of field grown hemizygous CG00554 x CG00526-176 plants at anthesis. Whole plants of field grown homozygous CG00526-176 inbreds at anthesis, and hemizygous CG00664 x CG00526-176 hybrids at seed maturity, were analyzed in these studies.

Leaf and Whole Plant Extraction Protocol. Three to four g of the powdered leaf or whole plant tissue (prepared as described in **Plant processing** in the main test of this volume) was extracted in two volumes of extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride HCl, 1 mM leupeptin, pH 10.0) using a Polytron® homogenizer (Brinkman, Westbury, NY). The homogenized material was filtered through cheesecloth which had been prewet with extraction buffer. The filtrate was centrifuged at 10,000 x g for 10 min. The supernatant was retained and its volume was measured. Solids which were retained by the cheesecloth, together with the pellet from the centrifugation step, were re-extracted in half the original volume of extraction buffer with or without 0.1% CHAPS detergent (Sigma, St. Louis, MO), filtered and centrifuged as before. For leaves only, after the second extraction, a third extraction was performed by grinding the remaining solids with a pestle, in a denaturing buffer containing 125 mM Tris, 10% b-mercaptoethanol, 4% SDS, 20% glycerol, and 0.002% bromophenol blue, and heating for 10 min at 65°C. The first two extracts were analyzed for CryIA(b) levels by enzyme-linked immunosorbent assays (ELISA) as described in Appendix A. Components of the denaturing buffer in which the third extract was prepared precluded the use of ELISA to analyze CryIA(b) protein levels; therefore, all three extracts were also analyzed by western blot analysis as described below. After treatment with iodoacetamide as described by Hill and Straka (1988), total protein in the extracts was determined using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL). Ovalbumin was used as the protein standard. Three different volumes of the sample aliquot were assayed to obtain at least two absorbance readings that coincided with the standard curve; the mean of these readings was used to calculate total protein. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu, Columbia, MD). Total protein could be quantified only for the first two extracts because of interference from the denaturing buffer in which the third extract was prepared.

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[2]"CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176. Lines designated by a single number with a "-176" suffix (e.g., "CG00526-176") are inbred lines that are homozygous for the transgenes.

Pollen Extraction. Pollen extracts were prepared from lyophilized pollen suspended 1:30 in extraction buffer. After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi, and centrifuged at 14,000 x g for 5 min at 4°C. The resulting pellet was resuspended in 2.5 ml fresh extraction buffer and French pressed as before. Total protein in the extracts was determined as above.

CryIA(b) Western Blot Analysis. Aliquots of the three sequential extracts prepared from leaf material were subjected to electrophoresis on a 10% polyacrylamide SDS gel (Novex, San Diego, CA). The first two extracts were diluted by an equal volume of 2X Laemmli sample buffer (20% glycerol, 2% b-mercaptoethanol, 4% SDS, 0.13 M Tris-HCl, 0.05% bromophenol blue, pH 6.8) and heated for 10 min at 65°C; the third extract was directly applied to the gel. The proteins were electroblotted onto Immobilon-P® membrane (Millipore, Bedford, MA) and developed using immunoaffinity purified rabbit antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD1 at a concentration of 0.133 mg antibody/ml blocking buffer (3% nonfat dry milk, 10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5). Goat anti-rabbit IgG linked to horseradish peroxidase (Bio-Rad, Hercules, CA), diluted 1:3000 in blocking buffer, was used to bind to the primary antibody and was detected using an ECL™ kit (Amersham, Arlington Heights, IL).

CryIA(b) Protein Quantification. The first two extracts were quantitatively analyzed for CryIA(b) protein by ELISA as described in Appendix A. No CryIA(b) was detected by western blot analysis in the third extract, therefore calculation of extraction efficiency was based on the total CryIA(b) recovered in the first two extracts. Total ng CryIA(b) in both extracts was determined and the efficiency of the initial extraction was calculated as:

$$\frac{\text{ng CryIA(b) in 1st extract}}{\text{ng CryIA(b) in 1st extract + 2nd extract}} \times 100 = \%\ \text{efficiency of first extraction}$$

$$\text{Example: } \frac{242.0\ \text{ng CryIA(b)}}{242.0 + 40.3\ \text{ng CryIA(b)}} \times 100 = 85.7\%\ \text{efficiency of first extraction}$$

RESULTS

The CryIA(b) protein present in the first leaf and pollen extracts represented approximately 87% and 85% of the total CryIA(b) extractable from those tissues, respectively. For whole plants the value was approximately 75% (Table 1). Inclusion of a detergent (0.1% CHAPS) in the second extraction buffer used with leaves had no significant effect on the percent CryIA(b) recovered in the second extract. Western blot analysis of SDS/heat extractable material remaining after the second extraction revealed no further detectable CryIA(b) associated with the insoluble material. This confirmed the efficacy of the standard methods employed in the first two extractions.

TABLE 4B-1: Extraction Efficiency of CryIA(b) from Leaves, Pollen and Whole Plants as Determined by ELISA

Exp. #.	Extraction	Leaves		Pollen		Whole Plant	
		ng CryIA(b)	% Efficiency*	ng CryIA(b)	% Efficiency	ng CryIA(b)	% Efficiency
1	First	242.0	85.7	135.0	88.4	384.9	75.3
	Second	40.3	14.3	17.8	11.6	125.9	24.7
2	First	1194.0	91.6	70.5	81.7	474.9	73.6
	Second	^109.0	8.4	15.8	18.3	170.1	26.4
3	First	4226.9	86.4			245.4	66.9
	Second	666.4	13.6			121.5	33.1
4	First	4024.1	86.4			355.8	81.6
	Second	^635.0	13.6			80.1	18.4
5	First	4081.2	87.3			281.4	76.8
	Second	594.1	12.7			85.2	23.2
6	First	4618.3	84.1				
	Second	871.4	15.9				
Mean of all experiments; 1st extraction			86.9		85.1		74.8

*% of total extractable CryIA(b), as calculated by:

$$\frac{\text{ng CryIA(b) in the extract}}{\text{ng CryIA(b) in first + second extract}} \times 100$$

^0.1% CHAPS included in second extraction buffer

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APPENDIX 4C

CryIA(b) Spike and Recovery Experiments

INTRODUCTION

Spike and recovery experiments were conducted to estimate the proportion of a purified CryIA(b) spike recoverable during the standard extraction procedures used for various maize tissues. The major tryptic fragment of native CryIA(b) was spiked into the extraction buffer used to extract nontransgenic control plant tissues (leaves, pollen, kernels and whole plants). Analysis was performed by ELISA to determine how much of the initial CryIA(b) spike was recovered.

MATERIALS AND METHODS

Plant Tissue Source. Mature leaves were taken from field grown nontransgenic CG00554 x CG00526 hybrid plants. Pollen samples were taken from greenhouse grown CG00526 inbred plants; kernels were obtained from field grown CG00526 inbred plants. Field grown CG00526 inbreds, harvested at anthesis, served as the source of whole plant material. Plant material was frozen in liquid nitrogen and maintained on dry ice or at -80°C until processed. Kernels were ground in a coffee grinder, leaves in a mortar and pestle, and whole plants in an ice grinder followed by a coffee grinder to yield a more fine powder.

Preparation of Tryptic Fragment of Native CryIA(b). The tryptic fragment of native CryIA(b), which is similar in size to the polypeptide encoded by the synthetic *cryIA(b)* gene used in Event 176 (approx. 607 vs. 648 amino acids) was purified from *Bacillus thuringiensis* subsp. *kurstaki* (*B.t.k.*) strain HD1-9 cell paste produced by fermentation under standard conditions. This strain was derived from strain HD1, the source of the native *cryIA(b)* gene upon which the synthetic gene in maize Event 176 was designed (Koziel *et al.*, 1993). Strain HD1-9 has been cured of plasmids such that the only Cry protein it produces is CryIA(b) (Carlton & Gonzalez, 1985, Minnich & Aronson, 1984). A 42 g aliquot of HD1-9 cell paste was resuspended in 200 mls 50 mM CAPS, 50 mM dithiothreitol, pH 10.0, and stirred on ice for 30 min. Spores were removed by centrifugation at 16,000 x g for 10 min at 4°C followed by centrifugation at 20,000 x g for 10 min. Trypsin (Fisher Scientific, Pittsburgh, PA; Cat. No. T360-500) was added to the supernatant (240 mg trypsin/approximately 200 ml supernatant) and the solution was stirred at room temperature for 2 h. The solution was then dialyzed against 50 mM sodium acetate, pH 5.2, (Spectra/Por7, 50,000 molecular weight cutoff, Fisher Scientific, Pittsburgh, PA) overnight at 4°C. The contents of the dialysis bag were centrifuged at 10,000 x g for 10 min at 4°C and the pellet was sequentially extracted by stirring on ice for 10 min in (1) 50 mM Tris-HCl, pH 7.5; (2) 50 mM Tris-HCl, pH 8.5; and (3) 50 mM CAPS, pH 10.0 and centrifuged as before. The supernatant of the final extraction contained approximately 2.5 mg total protein/ml and was highly enriched (as determined by SDS-PAGE) for the major tryptic fragment of CryIA(b). Total protein was quantitated by the Bio-Rad Coomassie blue method (Bio-Rad, Hercules, CA) using ovalbumin as the standard.

Tissue Extraction. All tissues were extracted following the same protocol except for pollen (described below). Three g of powdered tissue was extracted in 2 - 3 volumes of extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride HCl, 1 mM leupeptin, pH 10.0) using a

Polytron® (Brinkman, Westbury, NY). The homogenized material was filtered through cheesecloth which had been prewetted with extraction buffer. The filtrate was centrifuged at 10,000 x g for 15 min. The supernatant was retained and its volume was measured. This extract was used for CryIA(b) protein analysis by ELISA. After treatment with iodoacetamide as described by Hill and Straka (1988), total protein in the extracts was determined using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL) with ovalbumin as the protein standard. Three different sample sizes were assayed to obtain at least two absorbance readings that coincided with the standard curve; the mean of these readings was used to calculate total protein. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

Pollen extracts were prepared from lyophilized material suspended 1:30 in extraction buffer. After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi, followed by centrifugation at 14,000 x g for 5 min at 4°C. Protein was determined as described for the other tissues.

Spike and Recovery Procedure. Extraction was performed as described above in duplicate for each tissue with and without the prior addition of the purified tryptic CryIA(b) preparation to the extraction buffer such that the concentration of CryIA(b) ranged from 1 - 3 mg/ml. To verify the lack of interference by extracts during ELISA, an aliquot of the negative extract was also spiked with an equal concentration of the purified tryptic CryIA(b) fragment. The extraction buffers and the extracts were quantitatively analyzed for CryIA(b) by the standard ELISA protocol described in Appendix A.

CryIA(b) Protein Quantification. Extracts were quantitatively analyzed for CryIA(b) protein by ELISA as described in Appendix A. Recovery was calculated as:

$$\% \text{ recovery} = \frac{\text{ng CryIA(b)/ml extract}}{\text{ng CryIA(b)/ml spiked buffer}} \times 100$$

RESULTS

Recovery of purified CryIA(b) spiked into the extraction buffer prior to tissue extraction was different with each tissue examined and ranged from approximately 31% for leaves, 64% for kernels; 73% for whole plants to 100% for pollen (Table C-1). The relatively lower recovery in some tissues was not due to interference in the ELISA analysis as evidenced by the observation that when CryIA(b) was spiked directly into the tissue extract essentially all of it (94 - 114%, across all tissues; data not shown) was recovered by ELISA. Therefore, some CryIA(b) protein was lost to detection during the actual extraction process (e.g., perhaps by adhering to or having affinity for the cheesecloth, centrifuge tubes, plant material, etc.). Alternatively, this may have reflected inherent instability of CryIA(b) in different plant tissue extracts (e.g., through degradation by plant proteases) despite the inclusion of protease inhibitors in the extraction buffer.

TABLE 4C-1: Spike and Recovery of CryIA(b) Protein from Kernels, Leaves, Pollen and Whole Plants as Determined by ELISA

	% Recovery				
	Experiment 1		Experiment 2		
Tissue	Sample 1	Sample 2	Sample 1	Sample 2	Mean
Kernels^	64.2	64.9	61.9	na*	63.7
Leaves	30.3	32.2	27.8	31.9	30.6
Pollen	96.6	na	104.3	na	100.5
Whole Plants	81.8	64.2	na	na	73.0

^% moisture = 24
*not analyzed

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CHAPTER 5

Expression of Phosphinothricin Acetyltransferase in Maize Tissue

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5. Expression of Phosphinothricin Acetyltransferase in Maize Tissue

A. Summary

The range of expression of phosphinothricin acetyltransferase (PAT) in Ciba Seeds *Bt* maize plants derived from Event 176 was determined. This study involved the analysis of transgenic tissue samples using an enzyme-linked immunosorbent assay (ELISA) developed specifically for the PAT protein.

The following determinations were performed:

(1) PAT levels were examined in various plant tissues and developmental stages in three maize lines from 1993 field tests.

(2) stability of PAT expression over four successive backcross generations was examined in leaves and pollen of greenhouse grown plants representing two inbred maize lines;

(3) PAT levels were determined for selected tissues of four different greenhouse-grown *Bt* inbred maize lines;

B. Materials and Methods

PAT Quantification. The ELISA protocol for quantification of PAT protein in maize tissues and whole plants is detailed in Appendix 5A. Extraction efficiency and spike and recovery experiments were not conducted for PAT, because quantifiable levels of this protein were not detected in any tissue (see **RESULTS**).

Source of Plants for Developmental Study. PAT levels were determined for various plant tissues and developmental stages in three maize lines from 1993 field tests. Except for seedlings, which were greenhouse grown at the Ciba Agricultural Biotechnology Research Unit (Research Triangle Park, NC), all plants evaluated in this part of the study were field grown. Planting occurred on May 22, 1993, in Bloomington, Illinois, using standard agronomic practices. Transgenic plants from an inbred line, CG00526-176,[1] and two hybrid crosses, CG00554 x CG00526-176 and CG00664 x CG00526-176, were analyzed. Transgenic plants and the corresponding isogenic, nontransgenic controls were maintained concurrently in nearby plots subject to the same environmental conditions. Plants were harvested at four developmental stages:

(1) seedling, 5 - 6 leaf stage, three weeks after planting;

(2) anthesis (pollen shed), 10 - 11 weeks after planting;

(3) seed maturity, 18 - 20 weeks after planting; and

(4) senescence, 23 weeks after planting.

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[1] "CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176. CG00526-176 is an inbred line that is homozygous for the transgenes.

At each stage the entire plant with roots was harvested intact, bagged and labeled, and shipped overnight on wet ice to the Ciba Agricultural Biotechnology Research Unit. Upon receipt, several plants were separated into their respective tissues; the remainder were kept intact for later determination of whole plant levels of PAT protein. Total weight and leaf number were recorded for each plant. Plants were then stored at -80°C until processed and extracted (see below; **Plant processing** and **Tissue extraction**). The various tissue samples and whole plants were quantitatively analyzed for PAT levels by ELISA.

Source of Plants for Four Generation Study. The stability of PAT protein expression in leaves and pollen was evaluated in plants representing successive generations in the breeding process. Seed was collected from four successive backcross[2] generations (BC1, BC2, BC3, and BC4) derived by using line CG00526-176 as the source of the transgenes and either CG00554 or CG00642 as the recurrent parent. These seed were planted concurrently in the greenhouse and the seedlings were bioassayed against European corn borer (ECB), the target pest, to cull out negative segregants.[3] The remaining plants were grown in the greenhouse to anthesis at which time the uppermost leaves from each plant were removed. The tissue was extracted as described below in **Plant Processing** and **Tissue Extraction** and quantitatively analyzed for PAT protein by ELISA.

Plant Source for Inbred Comparisons. PAT levels were evaluated in selected tissues of four *Bt* maize inbred lines. The transgenes had been introgressed into these lines from the initial homozygous inbred, CG00526-176, by three successive backcrosses. Following self-fertilization, individual plants were identified as homozygous for the transgenes by bioassaying progeny of test crosses with nontransgenic plants against ECB and observing uniform insecticidal activity.[4] Mature leaves of confirmed homozygous, greenhouse-grown plants from lines CG00554-176, CG00615-176, CG00642-176, and CG00716-176 were harvested 13 weeks after planting and analyzed for PAT levels. Kernels were harvested from all four lines at seed maturity and also analyzed for PAT levels. PAT levels were additionally determined for the two lines for which pollen samples were available. Tissue extracts were prepared as described below (see **Plant processing** and **Tissue extraction**) and analyzed for PAT by ELISA.

Plant Processing. For all analyses of PAT protein in whole plants, frozen whole plants were reduced to a fine powder by grinding in an ice grinder followed by further grinding in a coffee grinder. The ground material was well mixed to ensure homogeneous sampling. Assessment of PAT protein in leaves was accomplished by crushing the entire complement of leaves in liquid nitrogen and mixing thoroughly. An aliquot of the tissue was ground further to a fine powder using a mortar and pestle. For all analyses of kernels, roots and pith, the entire frozen sample was ground to a fine homogeneous powder using a coffee grinder. During processing all samples were maintained in the frozen state by liquid nitrogen or dry ice. An aliquot was removed for lyophilization for dry weight determination. Samples for ELISA analysis were stored at -80°C until extracted.

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[2]Inbred conversion to introgress the *cryIA(b)* gene from one inbred line into a second required successive backcrosses using the second inbred as the recurrent parent.

[3]Negative segregants are progeny that, through Mendelian segregation, have not inherited the transgenes, despite having a transgenic parent(s).

[4]A cross of a homozygous *Bt+/Bt+* parent with a *Bt-/Bt-* (wild-type) parent yields only one progeny genotype, *Bt+/Bt-*. Because the *Bt* trait behaves as a dominant gene all progeny are thus insecticidal when challenged with ECB. In contrast, a cross of a hemizygous *Bt+/Bt-* parent with a *Bt-/Bt-* parent would produce 50% *Bt+/Bt-* (ECB active) and 50% *Bt-/Bt-* progeny.

Pollen was collected from individual tassels by homogenizing spikelets in a Waring blender (using two four-second pulses) in 50 ml extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride HCl, 1 mM leupeptin, pH 10.0). The pollen was separated from anther and tassel material by filtration through cheesecloth into parachute cloth. The pollen was washed off the parachute cloth into a centrifuge tube, the buffer was removed by aspiration after the pollen had settled, and the pollen was lyophilized.

Tissue Extraction. All tissues, except for pollen, were extracted as follows. Approximately 3 g of the powdered tissue was extracted in 2 - 3 volumes of extraction buffer using a Polytron® homogenizer (Brinkman, Westbury, NY). The homogenized material was filtered through cheesecloth which had been prewetted with extraction buffer. The filtrate was centrifuged at 10,000 x g for 15 min. The supernatant was retained and its volume was measured. This extract was used for PAT analyses by ELISA. After treatment with iodoacetamide as described by Hill and Straka (1988), total protein in the extracts was quantitated using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL). Ovalbumin was used as the protein standard. Three different volumes of each sample were assayed to obtain at least two absorbance readings that coincided with the standard curve. The mean of these readings was used to calculate total protein. Absorbance at 562 nm was monitored using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

Pollen extracts were prepared by suspending lyophilized pollen 1:30 (w/v) in extraction buffer. After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi, followed by centrifugation at 14,000 x g for 5 min at 4°C. Total protein was quantitated as described above.

C. Results

PAT Protein Levels During *Bt* Maize Development PAT protein levels were below the lower limit of quantification in all tissues analyzed for the three maize lines sampled at different developmental stages. PAT was detectable at trace levels in some leaf, root, pith and whole plant samples (Tables 1 and 2). PAT was not detected in pollen or kernels. *Bt* maize expression of the *bar* gene, which encodes PAT, is driven by the CaMV 35S promoter which has been reported to be inactive in pollen (Guerrero and Crossland, 1993; Koziel *et al.*, 1993).

PAT Protein Levels in Four Successive Generations of *Bt* Maize Plants. Leaves of anthesis stage plants from all four backcross generations during inbred conversion of two *Bt* maize lines were analyzed for PAT (Table 3). PAT protein was present in leaves at trace levels across the four generations but was below the lower limit of quantification in all samples.

PAT Protein Levels in Additional Homozygous Inbred *Bt* Maize Lines. PAT protein was present at trace levels in leaves of all four inbred lines, but was below the lower limit of quantification in all samples (Table 4). This was consistent with results seen in the developmental study with three other *Bt* maize lines (see **PAT protein levels during *Bt* maize development**), PAT was undetectable in kernels and pollen.

D. References

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TABLE 1. PAT Protein Levels on a Fresh Weight Basis During *Bt* Maize Development, Summer 1993

Tissue	Geno-type	Maize Line	mean ng PAT/g fr. wt. (N) Seedling[1]	Anthesis	Seed Maturity	Senescence
Leaves	+/+	526	< 200 (3)	< 200 (4)	< 200 (4)	nd (2)
	+/-	554x526	< 200 (5)	nd (3)	nd (3)	nd (2)
	+/-	564x526	< 200 (3)	nd (2)	< 200 (2)	< 200 (2)
Roots	+/+	526	nd (2)	< 100 (4)	< 100 (4)	na
	+/-	554x526	nd (6)	< 100 (3)	< 100 (3)	na
	+/-	564x526	nd (1)	nd (3)	< 100 (2)	na
Pith	+/+	526	na	< 200 (4)	< 200 (4)	na
	+/-	554x526	na	nd (3)	< 200 (3)	na
	+/-	564x526	na	na	nd (3)	na
Pollen	+/+	526	---	nd (4)	---	---
	+/-	554x526	---	na	---	---
	+/-	564x526	---	nd (3)	---	---
Kernels	+/+	526	---	---	nd (4)	nd (5)
	+/-	554x526	---	---	nd (3)	nd (3)
	+/-	564x526	---	---	nd (2)	nd (2)
Whole Plants	+/+	526	nd (3)	< 200 (6)	< 200 (5)	< 200 (4)
	+/-	554x526	< 200 (6)	nd (3)	< 200 (4)	< 200 (2)
	+/-	564x526	< 200 (2)	nd (3)	nd (4)	< 200 (2)

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. PAT was considered nondetectable (nd) if the mean absorbance generated during ELISA did not exceed that of controls, corresponding to 0 ng PAT. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue. Plants that were homozygous or hemizygous for the transgenes were designated "+/+" or "+/-", respectively. Proprietary Ciba Seeds lines abbreviated as: 526 = CG00526-176; 554 x 526 = CG00554 x CG00526-176; 564 x 526 = CG00564 x CG00526-176.

--- = not applicable (tissue not available at this developmental stage); na = not analyzed.

[1]Seedlings were greenhouse grown and analyzed three weeks after planting; all other stages were field-grown.

TABLE 2. PAT Protein Levels on a Dry Weight Basis During Maize Development, Summer 1993

Tissue	Geno-type	Maize Line	mean mg PAT/g dry wt. (N)			
			Seedling[1]	Anthesis	Seed Maturity	Senescence
Leaves	+/+	526	< 1.50 (3)	< 0.75 (4)	< 0.40 (4)	nd (2)
	+/-	554x526	< 1.50 (5)	nd (3)	nd (3)	nd (2)
	+/-	564x526	< 1.50 (3)	nd (2)	< 0.40 (2)	< 0.30 (2)
Roots	+/+	526	nd (2)	< 0.90 (4)	< 0.90 (4)	na
	+/-	554x526	nd (6)	< 0.90 (3)	< 0.90 (3)	na
	+/-	564x526	nd (1)	nd (3)	< 0.90 (2)	na
Pith	+/+	526	na	< 1.60 (4)	< 0.85 (4)	na
	+/-	554x526	na	nd (3)	< 0.85 (3)	na
	+/-	564x526	na	na	nd (3)	na
Pollen	+/+	526	---	nd (4)	---	---
	+/-	554x526	---	na	---	---
	+/-	564x526	---	nd (3)	---	---
Kernels	+/+	526	---	---	nd (4)	nd (5)
	+/-	554x526	---	---	nd (3)	nd (3)
	+/-	564x526	---	---	nd (2)	nd (2)
Whole Plants	+/+	526	nd (3)	< 1.20 (5)	< 0.50 (5)	< 0.35 (4)
	+/-	554x526	< 2.30 (6)	nd (3)	< 0.50 (4)	< 0.35 (2)
	+/-	564x526	< 2.30 (2)	nd (3)	nd (4)	< 0.35 (2)

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. PAT was considered nondetectable (nd) if the mean absorbance generated during ELISA did not exceed that of controls, corresponding to 0 ng PAT. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue. Plants that were homozygous or hemizygous for the transgenes were designated "+/+" or "+/-", respectively. Proprietary Ciba Seeds lines abbreviated as: 526 = CG00526-176; 554 x 526 = CG00554 x CG00526-176; 564 x 526 = CG00564 x CG00526-176.

— = not applicable (tissue not available at this developmental stage); na = not analyzed.

[1]Seedlings were greenhouse grown and analyzed three weeks after planting; all other stages were field-grown.

TABLE 3

PAT Levels in Several Backcross Generations During Bt Introgression into Inbred Lines CG00642 and CG00554

Generation	N	mean PAT levels Leaves (at Anthesis) µg/g fr. wt	µg/g dry wt.
CG00642			
BC1[a]	8	< 0.20	< 0.75
BC2	3	< 0.20	< 0.75
BC3	4	< 0.20	< 0.75
BC4	3	< 0.20	< 0.75
CG00554 Recurrent Parent			
BC1	2	< 0.20	< 0.75
BC2	5	< 0.20	< 0.75
BC3	2	< 0.20	< 0.75
BC4	5	< 0.20	< 0.75

All values were determined by ELISA and were not corrected for efficiency of extraction. Control plants had ELISA values corresponding to 0 ng PAT/g fr. wt. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue.

[a] Backcross generation

TABLE 4

PAT Levels in Several Greenhouse Grown Homozygous *Bt* Maize Inbred Lines

Tissue/Stage	Inbred Line	N[1]	mean PAT levels (range) µg/g fr. wt	µg/g dry wt.
Leaves at Seed Maturity	CG00554-176	1	< 0.20	< 0.40
	CG00615-176	1	< 0.20	< 0.75
	CG00642-176	3	< 0.20	< 0.75
	CG00716-176	5	< 0.20	< 0.75
Kernels at Seed Maturity	CG00554-176	1	nd	nd
	CG00615-176	1	nd	nd
	CG00642-176	3	nd	nd
	CG00716-176	5	nd	nd
Pollen	CG00554-176	2	nd	nd
	CG00642-176	1	nd	nd

All values were determined by ELISA and were not corrected for efficiency of extraction or recovery. All control plants had ELISA values corresponding to 0 ng PAT/g fr. wt. PAT was considered nondetectable (nd) if the mean absorbance generated during ELISA did not exceed that of controls, corresponding to 0ng PAT. Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue.

[1] Total number of plants available for analysis

APPENDIX 5A

PAT ELISA Protocol

INTRODUCTION

Extracts were quantitatively analyzed for phosphinothricin acetyltransferase (PAT) protein by enzyme-linked immunosorbent assays (ELISA, Tijssen, 1985) using immunoaffinity-purified polyclonal rabbit and protein G-purified polyclonal goat antibodies specific for *Escherichia coli* expressed PAT[1]. The ELISA method described here was adapted from procedures initially optimized for leaf tissue. The protocol, conditions employed, limitations and calculations are described in detail.

MATERIALS AND METHODS

ELISA Buffers. Buffers used were the same as described for the CryIA(b) ELISA Protocol.

PAT ELISA Protocol.

Vinyl 96-well plates (Costar, Cambridge, MA) were pre-washed for two hours with 95% ethanol and dried overnight. Plates were then coated with immunopurified polyclonal rabbit antibodies (2 µg/ml in borate buffered saline) specific for PAT purified from *E. coli* and incubated overnight at 4°C. Plates were washed three times with wash buffer and blocked for 45 min at room temperature with blocking buffer. Plates were washed three times and triplicate samples of each tissue extract (appropriate dilutions prepared in diluent) were applied (total volume was 50 µl). Following incubation at 4°C for 2.5 h, the plates were washed three times and coated with protein-G purified polyclonal goat antibodies specific for PAT purified from *E. coli* (4.0 µg/ml diluent). Plates were incubated for 1.5 h at 37°C and then washed three times prior to coating with rabbit anti-goat alkaline phosphatase conjugated antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted in diluent to 2 µg/ml in diluent. Plates were incubated at 37°C for 1 hour, washed three times and phosphatase substrate (0.6 mg p-phenyl phosphate/ml phosphatase substrate buffer) added. Color was allowed to develop for 45 min and then stopped by addition of 3 N NaOH. Absorbance at 405 nm was determined using a SLT 340 ATTC multiwell plate reader (Tecan, Research Triangle Park, NC) and results were analyzed using the Tecan SLT Soft2000 Curve fitting program (log/log.it algorithm).

Temperatures and incubation times were critical as the ELISA was not designed to go to equilibrium. Standard curves were prepared using PAT purified from *E. coli*, diluted in diluent with a 1:25 addition of crude extract (2 µl/50 µl). Standard curves were run daily and usually on each plate. The linear range of the assay was between 15 and 60 ng PAT/ml. PAT was considered to be nondetectable if the mean absorbance obtained for the triplicate ELISA samples did not exceed that of a wild type control or a negative segregant. Inclusion of extracts from tissues of nontransgenic plants had significant effects on the linearity range and the mean absorbance of standards suggesting nonspecific binding of the antibodies to components of the tissue extracts. Therefore, extracts of nontransgenic plant tissues were included in the standard curves to correct for background absorbance. The lower limit of quantification, below which PAT may be detectable but not quantifiable, was determined for various plant tissues at different developmental stages. On a fresh weight basis there was little difference in this value between developmental stages for a given tissue. The most variable tissue was leaves, which had lower

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[1]The *bar* gene from pCIB3064 was cloned into the inducible, overexpression pET-3c® vector (Novagen, Madison, WI) in *Escherichia coli* strain BL21(DE3)pLysS. PAT protein as encoded in this vector is identical in amino acid sequence to that encoded by pCIB3064 with an additional N-terminal 11 amino acid T7-Tag™. PAT purified from *E. coli* cultures was used to generate antibodies and for ELISA standards.

limits of quantification ranging from 100 - 200 ng CryIA(b) per g fr. wt. The 200 ng/g fr. wt. value was designated as the lower limit of quantification and is shown in Table A-1. On a dry weight basis, however, different lower limits of quantification were applicable to most tissues at different developmental stages, due to varying moisture content of the tissues.

TABLE 5A-1: **Approximate Lower Limits of Quantification of PAT in *Bt* Maize Tissues Derived from Event 176.**

Tissue	ng PAT / g fr. wt.	µg PAT / g dry wt.			
	All stages analyzed	Seedling	Anthesis	Seed Maturity	Senescence
Leaves	200	1.50	0.75	0.40	0.30
Kernels	120	*	*	0.15	0.14
Pith	200	*	1.60	0.85	*
Roots	100	2.80	0.90	0.90	*
Pollen	1100	*	2.30	*	*
Whole Plants	200	2.30	1.20	0.50	0.35

*Tissue not analyzed at this stage.

$$\text{Lower limit of quantification} = \frac{\text{lower limit of standard curve}}{\text{g fr. wt./ml extract at max. conc. usable in ELISA}}$$

Sample Calculation:

$$\text{For kernels: } \frac{15 \text{ ng PAT/ml}}{0.126 \text{ g fr. wt./ml extract}} = 119 \text{ ng PAT/g fr. wt.}$$

(value was rounded up to 120 ng PAT/g fr. wt. for Table 1).

PAT was detected at trace levels in some tissues and not detected at all in others examined from plants derived from Event 176 (See Tables 1, 2, this volume). Therefore to assure validity of the PAT ELISA methodology, maize plants derived from other transformation events containing the same *bar* gene construct were analyzed for PAT levels by ELISA (Table 5A-2). PAT protein was detectable and quantifiable in both the hybrid and inbred backgrounds (the zygosity of *bar* was not determined for these plants but was assumed to be hemizygous). In these extracts PAT was determined to be stable at 4°C for at least 5 days. Range finder ELISA assays were performed to allow dilutions of extracts to be prepared such that the mean absorbance of three replicates closely coincided with the midpoint of the standard curve. Only analyses in which the coefficients of variance were less than 10% were accepted. To avoid background interference, samples contained no more than 20 µg total protein/well as determined using the BCA™ Protein Assay Reagent (Pierce, Rockford, IL), as described in **Tissue extraction** in the main text of this volume.

TABLE 5A-2: Comparison of PAT Levels in Different Maize Transformation Events

Maize Line - Event	SEEDLING-STAGE LEAVES ng PAT/g fr. wt.	ANTHESIS-STAGE LEAVES ng PAT/g fr. wt.
CG00526-176	nd*	< 200^
CG00554 x CG00526-176	< 200	nd
CG00526-438	6536	7558
CG00554 x CG00526-299	3483	3414

*not detectable; mean absorbance did not exceed that of negative control plant extracts
^Where trace amounts were detectable but not quantifiable, values are shown as less than (<) the lower limit of quantification determined for that tissue.

PAT calculations:

$$\frac{\text{ng PAT}}{\text{ml extract}} = \frac{\text{mean ng PAT}^2}{\text{ml}} \times \frac{50\ \mu\text{l/well}}{\mu\text{l extract/well}}$$

$$\frac{\text{ng PAT}}{\text{g fr. wt.}} = \frac{\text{ng PAT}}{\text{ml extract}} \times \frac{\text{ml extract}}{\text{g fr. wt.}}$$

$$\%\ \text{dry weight} = \frac{\text{dry wt}}{\text{fr. wt.}} \times 100$$

$$\frac{\text{ng PAT}}{\text{g dry wt.}} = \frac{\text{ng PAT}}{\text{g fr. wt.}} \times \frac{\text{g fr. wt.}}{\text{g dry wt.}}$$

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[2]Obtained from ELISA

CHAPTER 6

Analysis of ß-lactamase Expression in Maize Tissue

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6. Analysis of ß-lactamase Expression in Maize Tissue

A. Summary

ß-lactamase is the product of the ampicillin resistance gene (*bla*) that is present in the transformation vectors used to create event 176. Two independent experimental approaches were undertaken to investigate whether there was expression of this gene in maize tissue. There was no evidence of ß-lactamase expression in either leaf tissue or pollen from transformed maize.

B. Introduction

In Chapter 2 of this petition, the two plasmid vectors used to transform maize are described. The progenitor plasmid utilized for the creation of these vectors is pUC19 (Yanisch-Perron *et al.*, 1985). This plasmid harbors a single copy of the *bla* gene which encodes ß-lactamase. This enzyme cleaves the four-membered ß-lactam ring found in ampicillin (and related compounds) rendering the antibiotic inactive. Growth on ampicillin-containing medium is the basis for selection of bacteria that harbor plasmids that express the *bla* gene. *Bla* gene expression in these vectors is under the control of a bacterial promoter. This promoter, as is true for all bacterial promoters studied, is not recognized by eucaryotic RNA polymerases. This should preclude the expression of the *bla* gene in our transformed maize line.

Expression of *bla* in tissue from event 176 was examined by testing for ß-lactamase activity in leaf and pollen extracts as well as by northern analysis of leaf tissue using a *bla* gene-specific probe.

C. ß-Lactamase Enzyme Analysis

- **Leaf Extraction.** Leaves of 3-6 week old greenhouse grown hybrid *BT* maize plants were used for ß-lactamase assays. The leaves were deveined, frozen in liquid nitrogen, and ground using a mortar and pestle. This material was extracted in three volumes of cold extraction buffer (50mM CAPS, 0.1M NaCl, 2mM dithiothreitol, 2mM EDTA, 1mM 4-(2-aminoethyl)- benzenesulfonylfluoride HCl, 1µM leupeptin, pH 10) per g of tissue with a Polytron homogenizer (Brinkman, Westbury, NY). The extract was filtered through cheesecloth, centrifuged at 10,000 x g at 4°C for 20 min, and the supernatant used as the source of enzyme.

- **Pollen extraction.** Pollen extracts were prepared from lyophilized event 176 pollen suspended 1:30 in extraction buffer. After 30 min on ice, the pollen suspensions were disrupted by three passages through a French pressure cell at 15,000 psi, and centrifuged at 14,000 x g for 5 min at 4°C. The resulting pellet was resuspended in 2.5 ml fresh extraction buffer , French- pressed as before, and the resulting suspension used as the source of enzyme.

- **ß-lactamase Assay.** The procedure described by DeBoer *et al.*, (1991) was employed, in which the chromophore 7-(thienyl-2-acetamido)-3-[2-(4-N,N-dimethylaminophenylazo)pyridinium-methyl]-3-cephem-4-carboxylic acid (PADAC, Calbiochem, La Jolla, CA) was used as the substrate. The decrease in absorbance at 570 nm was monitored for one min using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

- **Protein Determination.** Following treatment with iodoacetamide (Hill and Straka, 1988), total protein was determined by the BCA procedure (Pierce, Rockford, IL) with ovalbumin used as the protein standard. Absorbance at 562 nm was measured using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

- **Isolation of *E. coli* expressed ß-lactamase.** *E. coli* strain BL21(DE3)pLysS was transformed with the vector pET-3c (Novagen, Madison, WI). This plasmid contains the identical *bla* gene present in our two transformation vectors. Cells were grown in Luria broth containing ampicillin (100mg/L), centrifuged and washed in 50mM Tris, 2mM EDTA, pH 7.5. The bacteria were frozen at -20° C, thawed and sonicated to lyse the cells. The samples were centrifuged and the supernatant used as the enzyme source.

D. Results of ß-lactamase Enzyme Assays

The *E. coli* extract exhibited a ß-lactamase specific activity of 0.14 mM/min/mg protein. There was no change in absorbance of the substate PADAC when either maize leaf extracts or pollen extracts were used as the enzyme source, regardless of the amount of sample assayed (up to 40-fold more protein than was assayed for *E. coli*). This indicates that there is no catalytically active ß-lactamase enzyme present in transformed leaf or pollen tissue.

E. Northern Analysis

- **Materials and Methods.** Total RNA was isolated from event 176 leaf tissue. One gram of freshly harvested tissue from three anthesis stage (ECB-bioactive; line CG00526-176) maize plants was individually processed for total RNA according to published procedures (Current Protocols in Molecular Biology, 1993. John Wiley & Sons, Inc.). RNA (20 µg/sample) was subjected to electrophoresis and blotted onto nylon membrane using standard procedures (Maniatis *et al.*, 1982). The blot was initially hybridized to a radioactively labelled *bla* gene fragment to detect ß-lactamase-specific messenger RNA. Following exposure to X-ray film, the membrane was treated to remove the radioactive probe and the blot was reprobed with a radiolabelled *cryIA(b)* gene fragment. This second probing with *cryIA(b)* was performed to ensure that the maize RNA was of sufficient quality and quantity to detect expression of a transgene. Two additional controls were performed. First, the *bla* probe was hybridized to pUC19 DNA (harboring a *bla* gene) to ensure the integrity of the probe used in the northerns. Second, a positive control northern was performed using total RNA from *E. coli* (transformed with pUC19) known to be expressing *bla* messenger RNA.

F. Results of Northern Analysis

There was no hybridization to RNA isolated from *cryIA(b)* transformed maize tissue using the *bla* gene as a probe (Figure 1, lanes 5-7). This same probe did hybridize to both the pUC19 plasmid control DNA (data not shown) as well as to RNA isolated from bacteria expressing the identical *bla* gene present in our transformed maize plants (lane 10). Using the *cryIA(b)* gene fragment as a probe, a strong signal was seen in all three lanes representing transformed maize lines (lanes 1-3). There was no hybridization to RNA isolated from untransformed maize tissue using either probe (lanes 4, 8).

The lack of detectable *bla* messenger RNA from transgenic leaf tissue indicates that the *bla* gene is transcriptionally inactive. This result, coupled with the ß-lactamase enzyme data, indicates that expression of the *bla* gene in transformed maize tissue is highly unlikely.

G. References

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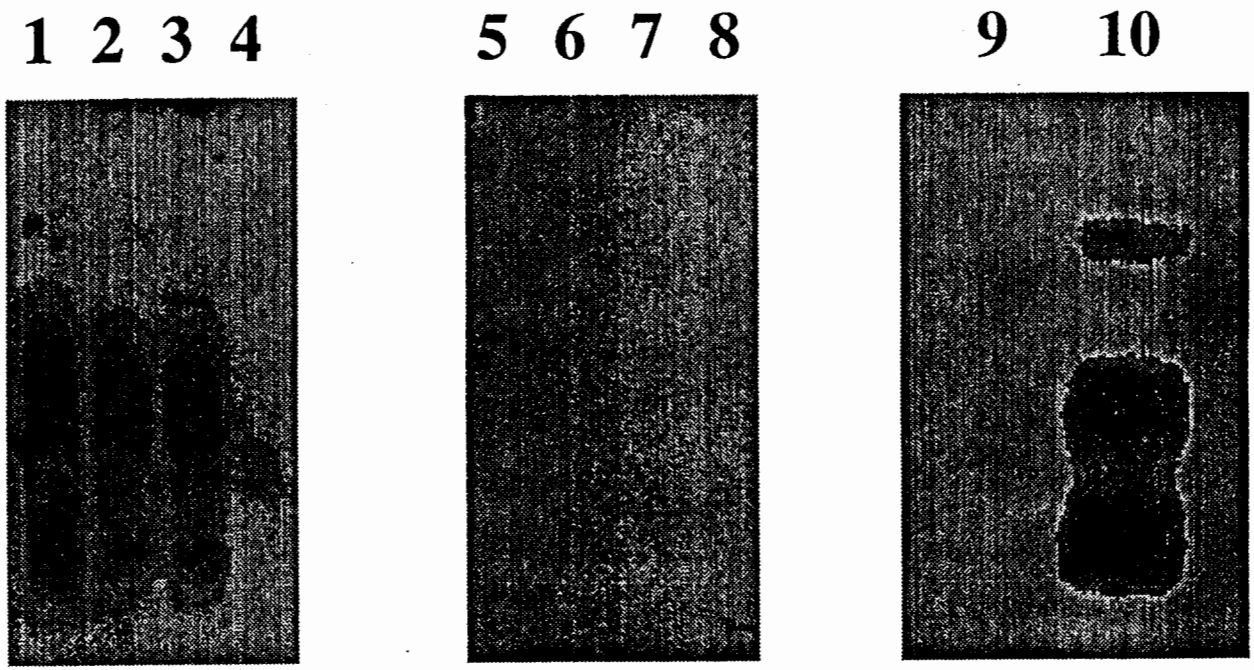

Figure 1. Northern Analysis of RNA from Event 176-Derived Maize Plants

Northern blots were performed to determine expression of the procaryotic *bla* gene in event 176-derived maize tissue. 20 µg of total RNA from each of three transformed plants (Bt homozygous; Southern positive for the *bla* gene) and a non-transformed control plant was electrophoresed, transferred to nylon membrane, and sequentially hybridized to a ^{32}P-labelled *bla* gene fragment and a ^{32}P-labelled *crylA(b)* gene fragment. Lanes 5-7: RNA from event 176-derived plants hybridized with *bla* gene probe; Lanes 1-3: identical blot hybridized with *crylA(b)* gene probe; Lanes 4, 8: RNA from non-transformed maize tissue hybridized to *crylA(b)* probe, or *bla* gene probe, respectively; Lane 9: RNA isolated from *E. coli* strain BL21 probed with *bla* gene fragment; Lane 10: RNA isolated from *E. coli* strain BL21 harboring the vector pET-3c, probed with the *bla* gene.

CHAPTER 7

In Vitro Digestibility and Inactivation of the *Bar* Marker Gene Product Phosphinothricin Acetyltransferase (PAT) Under Simulated Mammalian Gastric Conditions

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7. *IN VITRO* DIGESTIBILITY AND INACTIVATION OF THE *BAR* MARKER GENE PRODUCT PHOSPHINOTHRICIN ACETYLTRANSFERASE (PAT) UNDER SIMULATED MAMMALIAN GASTRIC CONDITIONS

A. Summary

The susceptibility of phosphinothricin acetyltransferase (PAT) to inactivation and proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin. PAT degraded extremely rapidly when pepsin was present at the standard concentration in SGF. Furthermore, PAT enzymatic activity was immediately and irreversibly lost at 37°C in SGF with or without pepsin. These data suggest that PAT will likely lose enzymatic activity immediately at gastric pH (1.0 - 1.2) and will be digested as conventional dietary protein in the typical mammalian gastric environment.

B. Introduction

The purpose of this study was to determine whether PAT enzymatic activity is lost and the protein readily degraded under simulated mammalian gastric conditions. Results of this study regarding enzymatic inactivation parameters and degradation potential of PAT protein are relevant to the overall safety assessment of *Bt* maize, as the primary route of exposure to transgenic proteins in corn would be dietary.

C. Materials and Methods

- **Source of PAT.** PAT expression was found to be quite low in maize plants derived from transformation event 176 (see Chapter 5 of this petition). Due to this low expression it was not possible to extract sufficient amounts of PAT from the *Bt* maize plants for this study. The PAT protein utilized in these studies was purified from an *Escherichia coli* expression system. The *bar* gene from pCIB3064 was cloned into the inducible, overexpression pET-3c® vector (Novagen, Madison, WI) in *E. coli* strain BL21(DE3)pLysS. PAT protein as encoded in this vector is identical in amino acid sequence to that encoded by pCIB3064 except for an additional N-terminal 11 amino acid T7-Tag™.

- **PAT Purification.** Expression of the *bar* gene was induced in cultures of the recombinant *E. coli* by the addition of 1 mM isopropylthiogalactopyranoside (IPTG, Sigma, St. Louis, MO). After four hours, cells were harvested by centrifugation and washed with ice cold extraction buffer (50 mM Tris-HCl, 2 mM EDTA, pH 7.5). Pellets were frozen until utilized. Cell pellets were thawed and resuspended in extraction buffer at 1/10th the original culture volume. The cells were lysed by sonication and the debris was removed by centrifugation at 16,000 x g for 10 min at 4°C. The supernatant volume was measured and brought to 30% saturation with ammonium sulfate (16.4 g/100 mls) by slow addition of crystalline ammonium sulfate with stirring at 4°C. The precipitate was collected by centrifugation at 10,000 x g for 20 min at 4°C. The pellet was resuspended in 50 mM NH_4HCO_3, pH 7.8, and desalted over an Econo PAC™ 10DG column (Bio-Rad, Hercules, CA) equilibrated in the same buffer and maintained at 4°C. Analysis by SDS polyacrylamide gel electrophoresis (SDS-PAGE) indicated that PAT represented approximately 90% of the protein present in this sample, which was used in the digestibility studies outlined

below. Protein content of this sample was determined to be 2.6 mg/ml by the BCA™ procedure (Pierce, Rockford, IL) using ovalbumin as the standard.

- **Simulated Gastric Fluid.** Simulated mammalian gastric fluid (SGF) containing NaCl (2 mg/ml), HCl (14 µl 6N HCl/ml) and pepsin (3.2 mg/ml, 3200-4500 units/mg) (Sigma, St. Louis, MO), was prepared fresh daily as described in the United States Pharmacopoeia (1990). The pH of the SGF was 1.0 to 1.2 and was not further adjusted. Initial studies indicated degradation of PAT to be so rapid when SGF was prepared at the standard pepsin concentration (1X; 3.2 mg/ml) that SGF was also prepared with pepsin at decreasing concentrations (0.1X, 0.01X, and 0.001X the standard concentration). SGF without pepsin (0X) was also prepared. Prior to all digestibility experiments, as a positive control, the activity of each freshly prepared batch of SGF (standard pepsin concentration) was verified using azoalbumin (Sigma, St. Louis, MO), a substrate that upon proteolysis releases 10% trichloroacetic acid soluble color which was monitored at 389 nm using a UV160U spectrophotometer (Shimadzu, Columbia, MD).

- **Pepsin Concentration Digestion Experiments.** These experiments were conducted to evaluate the degradation of PAT in the presence of the standard SGF, as well as in lower concentrations of pepsin. Digestions were set up with PAT protein samples prepared as described above (see **PAT Purification**). SGF was prepared at pepsin concentrations of 1X, 0.1X, 0.01X, and 0.001X the standard concentration (see **Simulated Gastric Fluid**). SGF was also prepared without pepsin (0X). Each reaction was initiated by the addition of 10 µl of PAT sample (containing 26 µg of total protein) to 90 µl of the appropriate SGF solution. After vortexing, 50 µl was removed, immediately neutralized by an equal volume of 2X Laemmli SDS sample buffer (20% glycerol, 2% b-mercaptoethanol, 4% SDS, 0.13 M Tris-HCl, 0.05% bromophenol blue, pH 6.8), brought to 75°C, and heated for 10 min. This was designated the time zero sample. The remainder was incubated at 37°C for 2 min prior to being neutralized and heated in the same manner as the time zero sample.

- **SDS-PAGE Analysis of PAT.** A 30 µl aliquot was taken from each reaction sample collected during PAT digestibility experiments and was subjected to SDS polyacrylamide gel electrophoresis (SDS-PAGE) on 14% polyacrylamide gels (Novex, San Diego, CA). After staining with Coomassie blue and destaining, gels were examined for presence of the PAT protein, approximately 22,000 mol. wt.

- **Enzymatic Inactivation Studies.** The stability of PAT enzymatic activity was studied in several ways: (1) To assess thermolability of PAT at pH 7.5, the pH optimum for the enzyme (Botterman *et al.*, (1991), PAT activity was measured before and after incubation in PAT assay mix (described below) at 37°C for 10 min; (2) To measure PAT enzymatic stability at gastric pH (1.0 - 1.2), samples were removed for enzyme assays throughout a 10 min incubation of PAT in SGF without pepsin at 37°C; and (3) To measure the sensitivity of PAT activity to degradation by pepsin in SGF, samples were removed for enzyme assays throughout a 10 min incubation of PAT in SGF with 0.001X the standard pepsin concentration at 37°C. Experiments were designed to analyze samples from the same reaction mix for enzymatic activity by PAT assays and for protein integrity as visualized by SDS-PAGE. Reactions were initiated by addition of 40 µl of PAT sample to 360 µl of 0.001X pepsin in SGF. Parallel reactions were run in SGF without pepsin (0X). At time 0, and after 0.5, 1, 2, 5, and 10 min of incubation at 37°C, two 25 µl aliquots were removed. One aliquot was neutralized by addition of 25 µl Laemmli SDS sample buffer (see **Pepsin**

Concentration Digestion Experiments) and heated at 75°C. These samples were subjected to SDS-PAGE and the resulting stained gels were analyzed densitometrically using a GDS7500 Gel documentation and Gelbase/Gelblot analysis system (UVP, Upland, CA). The other aliquot was immediately added to PAT assay mix (0.5 mM acetyl CoA, 0.4 mg dithiobisnitrobenzoic acid (DTNB), 20 mM Tris-HCl, pH 7.5) and placed on ice until assayed. To determine whether inactivation was occurring due to the gastric conditions or to exposure of PAT to 37°C, an aliquot of PAT was incubated in assay mix, at pH 7.5, the optimum for PAT, at 37°C for 10 min prior to being assayed and its activity compared to a similar aliquot which had remained on ice. PAT assays were initiated by the addition of phosphinothricin to the assay mix at a final concentration of 1 mM. PAT catalyzes the transfer of the acetyl group from acetyl CoA to phosphinothricin. This releases a free thiol which reacts with DTNB, generating a yellow color the absorbance of which was monitored at 412 nm using a UV160U spectrophotometer (Shimadzu, Columbia, MD). One unit of PAT activity is defined here as one mmole DTNB reduced per min at ambient temperature.

D. RESULTS

- **Pepsin Concentration-Dependent Digestion.** In the presence of SGF at the standard pepsin concentration (1X), the PAT protein was completely degraded at time zero as visualized after staining a polyacrylamide gel (Figure 1). After 2 min in SGF prepared with 0.001X the standard pepsin concentration, a significant amount of PAT had not been degraded. Therefore, this pepsin concentration was selected for the inactivation studies.

- **Enzymatic Inactivation Studies.** The initial specific activity of the PAT sample used in these experiments was 10.5 U/mg protein (27.0 U/ml); however, after incubation at 37°C in assay mix for 10 min, activity decreased to 5.9 U/mg protein (15.2 U/ml) or 56% of the control value. This decrease reflects the thermal sensitivity of the enzyme above 35°C as has been reported previously (Botterman *et al.*, 1991). This represented the maximum activity that would have been recoverable if exposure to gastric pH had no effect on PAT activity or if its effect were reversible. However, immediately upon addition to SGF without pepsin (time zero), PAT activity decreased to 0.7 U/ml, corresponding to only 2.6% of the initial activity, and activity reached zero by one min (Figure 2A). The time zero value was even lower when PAT was exposed to 0.001X the standard pepsin concentration in SGF (Figure 2B). Activity was not restored by neutralization, indicating irreversibility of the enzymatic inactivation. Densitometric analysis of PAT, approximately 22,000 mol. wt., indicated an apparent half-life for degradation in 0.001X the standard pepsin concentration in SGF of between 1 and 2 min (Figure 2B).

E. Discussion

Jones and Maryanski (1991), in their discussion on safety considerations in the evaluation of transgenic plants for human food, make several points on the important differences between industrial chemicals and novel proteins in transgenic food plants. They note that:

(1) Proteins are usually acutely nontoxic, and there are no known proteins that exhibit chronic toxicities such as mutagenesis or carcinogenesis. Those proteins that are toxic are highly specialized and well characterized;

(2) Proteins are usually degraded by digestion in the mammalian alimentary tract;

(3) Proteins do not appear to be bioaccumulated as are some chemicals;

(4) The biological activity of the protein is important; whether the protein retains its activity in the mammalian alimentary tract should be considered.

Protein digestion in humans and higher animals is initiated by proteases, predominantly pepsin, that are secreted by the epithelial cells of the stomach lining. These proteases are optimized for this low pH (pH 1-2) environment. Hydrolysis by proteases produces a mixture of peptides and amino acids, which in turn are potent stimulators of gastric action (Alpers, 1987). The stomach has a normal half clearing time for liquids of 12 to 64 minutes. For solids the range is between 45 and 195 minutes (Yamada, 1991). Additional proteolysis occurs in the intestines at above-neutral pH, where multiple proteases of wide ranging specificities are found. Proteins are not usually absorbed in the intestines in their intact form, but rather as their amino acid components and small peptides (Alpers, 1987; Fuchs *et al,* 1993).

Proteins which are not easily digested may be absorbed in small quantities and serve as antigens resulting in allergic responses. Common examples include casein in milk and glutens from wheat. Both of these proteins are relatively resistant to enzymatic digestion (Alpers,1987).

The results of this study indicate that PAT loses enzymatic activity immediately upon exposure to gastric pH and that PAT protein is readily degraded in a simulated mammalian gastric environment. At the standard pepsin concentration, the protein was degraded so rapidly that no PAT could be detected upon immediate sampling of the reaction mix. Significant dilution of pepsin (0.001X) was required for a half-life of 1 - 2 min of the approximately 22,000 mol. wt. PAT protein, as measured by densitometric scanning of a Coomassie blue-stained gel. Furthermore, no enzymatic activity of PAT was detectable by 1 min in the simulated mammalian gastric fluid, with or without pepsin. These data suggest that maize produced PAT protein will not retain enzymatic activity and will be readily digested in the mammalian gastric environment as conventional dietary protein in the unlikely event of dietary exposure. Its potential for allergenicity appears extemely remote, given its rapid proteolytic degradation.

In conclusion, expression of PAT in Ciba Seeds' *Bt* maize Event 176 should pose no dietary risk to humans, domestic animals, or wildlife due to the following combination of factors:

1. PAT bears no homology to any known toxic proteins (Genbank version 7.1 search);

2. The mode of action of the PAT enzyme is well characterized, and its mode of action does not resemble that of any known protein toxin.

3. PAT enzyme is immediately inactivated at low pH at 37°C in the typical mammalian gastric environment;

4. PAT undergoes rapid proteolytic degradation in the typical mammalian gastric environment;

5. PAT protein levels are extremely low in maize plants derived from Event 176. There are no detectable residues in kernels or pollen;

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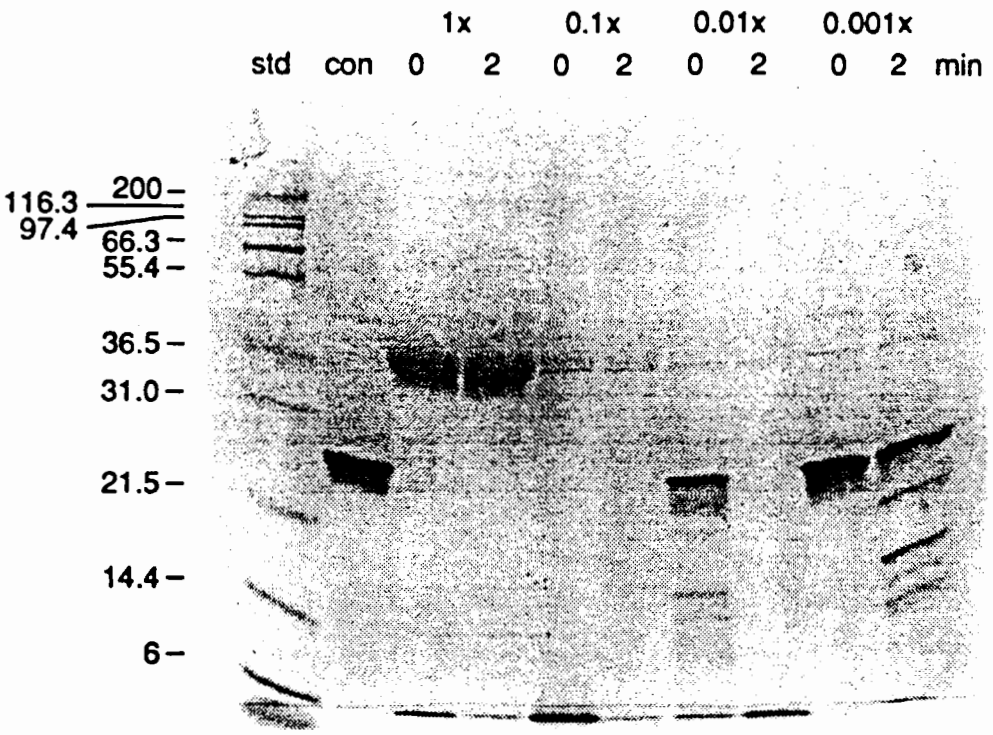

Figure 1. Digestion of PAT Protein (approx. 22,000 mol. wt.) in Simulated Gastric Fluid (SGF) Prepared at Various Concentrations of Pepsin.

Digestion reactions were prepared and PAT degradation was visualized via Coomassie blue staining following SDS-PAGE as described in Materials and Methods. SGF was used at the standard concentration of pepsin (1x) or prepared with 0.1x, 0.01x, and 0.001x the standard pepsin concentration. SGF prepared without pepsin was designated 'con'. Each lane represents a sample that initially contained 4 mg of PAT protein. The intensely staining band at an apparent molecular weight of 35,000 in 1x SGF is pepsin. Molecular weight standards ($x10^{-3}$) are indicated.

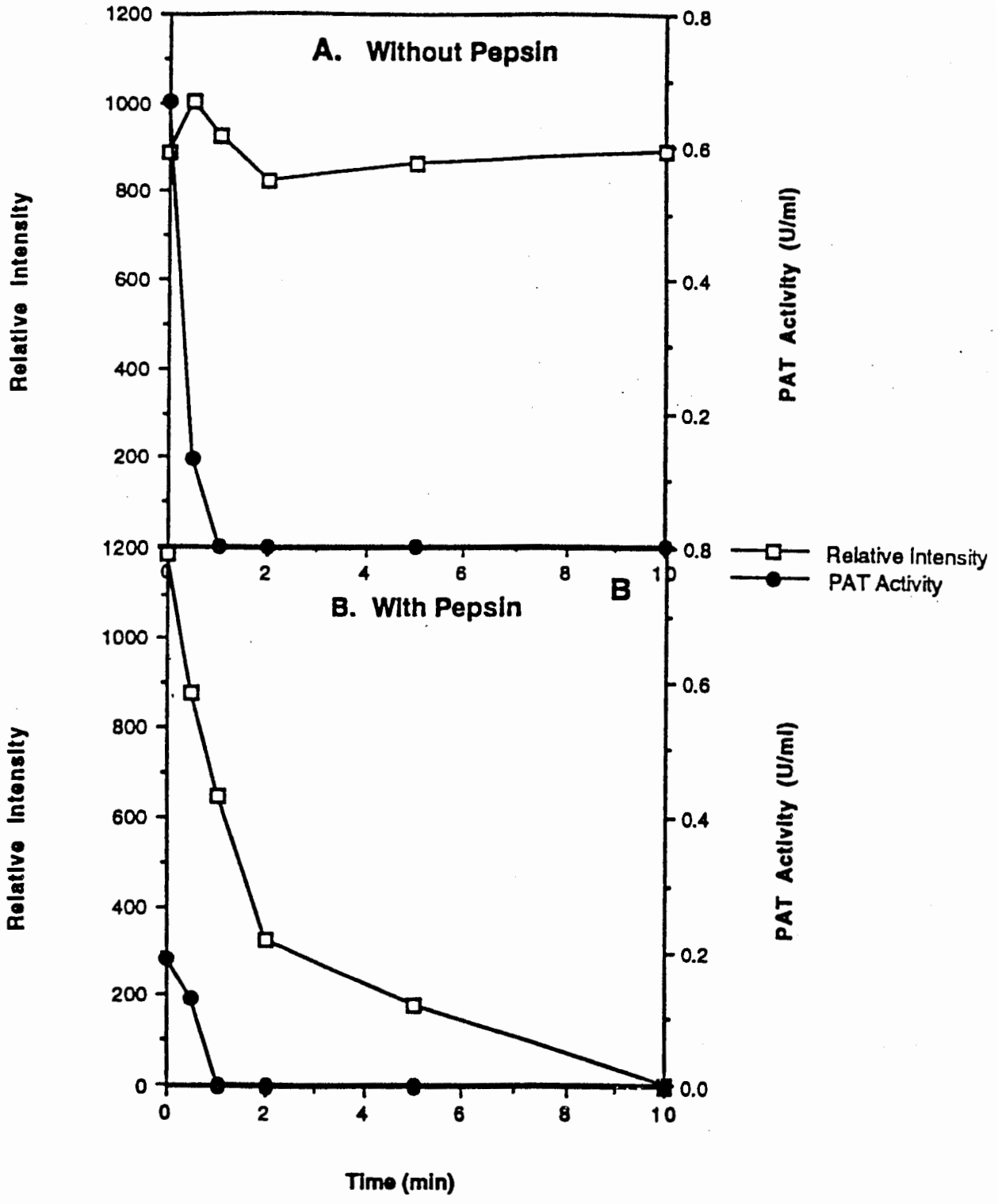

Figure 2. Inactivation of PAT in 0.001X Pepsin in Simulated Gastric Fluid

Reactions were prepared as described in Materials and Methods without pepsin (Panel A) or with 0.001X the standard pepsin concentration (Panel B) in simulated gastric fluid. Densitometric analysis of samples subjected to SDS-PAGE on 14% polyacrylamide gels yielded relative intensities for PAT at each time point (-□-). The results of parallel samples assayed for PAT activity are also shown for each time point (-•-).

CHAPTER 8

In Vitro Digestibility and Acute Oral Toxicity of CryIA(b) Protein Derived from *Bt* Maize and *Bacillus thuringiensis* subspecies *kurstaki*

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8. *IN VITRO* DIGESTIBILITY AND ACUTE ORAL TOXICITY OF CRYIA(B) PROTEIN DERIVED FROM *BT* MAIZE AND *BACILLUS THURINGIENSIS* SUBSPECIES *KURSTAKI*

A. Summary: *in vitro* digestiblity studies in a simulated mammalian gastric environment

The susceptibility of maize-expressed CryIA(b) protein to proteolytic degradation was evaluated in simulated mammalian gastric fluid (SGF) containing pepsin. As a comparison, the native CryIA(b) protein from *Bacillus thuringiensis* subsp. *kurstaki* was also tested. Maize-expressed CryIA(b) was rapidly degraded in SGF such that no immunoreactive CryIA(b) polypeptides were detectable by western blot analysis upon immediate sampling of the reaction mixture. In order to demonstrate a time-course of maize CryIA(b) degradation, the pepsin concentration in the SGF was reduced to 0.001X the standard concentration; under these conditions maize CryIA(b) polypeptides were undetectable by western blot analysis after 10 min. Native CryIA(b) was also rapidly degraded in the standard SGF; when the pepsin was reduced to 0.01X the standard concentration, degradation occurred within 5 min. These data suggest that maize-expressed CryIA(b) protein will likely be digested as conventional dietary protein in the typical mammalian gastric environment.

B. Introduction

The purpose of this study was to determine whether maize-expressed CryIA(b) is readily degraded under simulated mammalian gastric conditions. For comparison, the *in vitro* digestibility of native CryIA(b) protein from *B.t.k.* was also evaluated. In addition to examining the degradation of CryIA(b) protein under standard assay conditions, additional experiments were conducted to demonstrate a time-course of CryIA(b) degradation. These experiments required significant dilution of the pepsin concentration in the simulated gastric fluid.

Results of this study are relevant to the overall safety assessment of *Bt* maize, as the primary route of exposure to transgenic proteins in corn would be dietary.

C. Materials and Methods

- **Source of Maize-Expressed CryIA(b).** To obtain a relatively concentrated sample of maize-expressed CryIA(b) protein for analysis, *Bt* maize leaf protein was extracted and enriched for CryIA(b). *Bt* maize leaves were obtained from mature field-grown hybrid plants of cross CG00554 x CG00526-176[1]. Leaves were collected on 2 Sep 93 in Bloomington, IL and on 2 Aug 93, 20 Aug 93 and 30 Aug 93 in Research Triangle Park, NC. The leaves collected in Bloomington were shipped overnight on wet ice to the Ciba Agricultural Biotechnology Research Unit, Research Triangle Park, NC. Upon receipt from Bloomington or collection in Research Triangle Park, the leaves were deveined, quick frozen and crushed in liquid nitrogen. The crushed leaves were stored at -80°C until processing, which was initiated on 12 Nov. 93.

[1]"CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176. CG00526-176 is an inbred line that is homozygous for the transgenes.

- The crushed leaf material was powdered in a mortar and pestle in liquid nitrogen and then stored at -80°C or extracted immediately by homogenizing 400-600 g batches in three volumes of 4°C extraction buffer (50 mM CAPS, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1 mM leupeptin, pH 10.0) per unit weight of leaf powder. Homogenization was done in a precooled CB6 Waring Industrial blender fitted with a double blade. All further processing of the extracts was conducted at 4°C or by maintaining the materials chilled on ice. Each extract was filtered through cheesecloth and the solids retained by the cheesecloth were re-extracted in one-half the original volume of extraction buffer, filtered through cheesecloth and pooled with the original extract. The total volume was measured and 10% polyethylenimine was added to a final concentration of 0.15%. Extracts were centrifuged at 7280 x g (in a Sorvall RC3-B centrifuge, H600A rotor) for 20 min. The supernatant was filtered through miracloth (Calbiochem, San Diego, CA) and its volume was measured and brought to 40% saturation with ammonium sulfate (22.4 g/100 ml) by slow addition of crystalline ammonium sulfate with stirring. The precipitate was collected by centrifugation at 7280 x g for 20 min. The pellet was resuspended in one-tenth the original volume of the pooled extracts in 50 mM NH_4HCO_3, pH 10.0, by stirring gently until dissolved. Remaining particulates were removed by centrifugation at 7280 x g for 20 min. The supernatant was desalted by dialysis (Spectra/Por7 dialysis tubing, 50,000 mol. wt. cutoff, Fisher Scientific, Pittsburgh, PA) against 50 mM $NaHCO_3$, pH 10.0, (24 L/1.5 - 2 L extract) without stirring for 30 min to 2 h followed by continuous flow (50 ml/min) dialysis against 64 L 50 mM NH_4HCO_3, pH 10. The contents of the dialysis bags were collected, shell frozen and lyophilized to yield a tan fluffy powder. CryIA(b) represented approximately 0.07% of the resulting test material by weight as determined by ELISA. The CryIA(b)-enriched lyophilized leaf protein preparation was dissolved in distilled water at 10 mg/ml for use in *in vitro* digestibility studies.

- **Source of Native CryIA(b).** *B.t.k.* strain HD1-9 has been cured of plasmids such that the only Cry protein it produces is CryIA(b) (Carlton and Gonzalez, 1985; Minnich and Aronson, 1984). This strain was derived from *B.t.k.* strain HD-1, the source of the native *cryIA(b)* gene upon which Ciba Seeds' synthetic gene in corn was designed to encode in truncated form. From 28 Apr. 93 - 7 May 93, three 250-L batches of *B.t.k.* strain HD1-9 were prepared by the fermentation facility at the University of Minnesota and the sporulated cells were pooled and concentrated using standard techniques. The resulting cell paste was assigned sample no. UM305006 by the fermentation facility. The cell paste was frozen in approximately 200-g aliquots and shipped overnight on dry ice to the Ciba Agricultural Biotechnology Research Unit, Research Triangle Park, NC, where it was received on 14 May 93 and re-assigned the sample lot. no. HD19-UM305006. The cell paste was stored at -20°C until used.

- A 200 g aliquot of HD1-9 cell paste was resuspended in 1L 0.05% Triton X-100 and centrifuged at 7280 x g in a Sorvall RC3-B centrifuge (H600A rotor) for 20 min at 4°C. The pellet was washed twice in 1 L of 50 mM Tris-HCl, 0.2 M NaCl, 2 mM EDTA, pH 8.0, and centrifuged as above. The endotoxin crystals were solubilized in 2 L 50 mM CAPS, 25 mM b-mercaptoethanol, pH 10.0, and the spores were immediately removed by centrifugation as before. The supernatant containing solubilized crystals was retained and any crystals remaining in the pellet were solubilized in 1 L 50 mM CAPS, 25 mM b-mercaptoethanol, pH 10.0, and spores removed by centrifugation. Sodium acetate (3 M, pH 5.2) was added to the solubilized crystals to a final concentration of 10 mM. The pH of the solution was lowered to pH 5.1 - 5.5 with 6 N HCl causing precipitation of CryIA(b) protein. This precipitate was collected by centrifugation at 10,000 x g for 15 min and washed twice with 10 mM

sodium acetate, pH 5.2. Following centrifugation at 10,000 x g for 15 min, the resulting pellet was lyophilized overnight. The CryIA(b) protein preparation was resuspended at 10 mg/ml in distilled water and sonicated; this solution was used for the *in vitro* digestibility assays. Full length CryIA(b) (approximately 128,000 mol. wt.) represented approximately 46% of the protein [as determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)] present in the preparation.

- **Simulated Gastric Fluid.** Simulated mammalian gastric fluid (SGF) was prepared as described in Chapter 7 of this petition (United States Pharmacopoeia, 1990).

- **Pepsin Concentration Digestion Experiments.** These experiments were conducted to evaluate the degradation of maize CryIA(b) and native CryIA(b) in the presence of the standard SGF, as well as to identify an appropriate pepsin concentration for use in later time-course experiments. Digestions were set up with both maize-expressed and native CryIA(b) protein samples prepared as described above (see **Source of maize-expressed CryIA(b)** and **Source of native CryIA(b)**). SGF was prepared at pepsin concentrations of 1X, 0.1X, 0.01X, and 0.001X the standard concentration (see **Simulated gastric fluid**). SGF was also prepared without pepsin (0X). Each reaction was initiated by the addition of 10 µl of sample (the maize-expressed CryIA(b) or native CryIA(b)), containing 100 µg total protein to 90 µl of the appropriate SGF solution. After vortexing, 50 µl was removed, immediately neutralized by an equal volume of 2X Laemmli SDS sample buffer (20% glycerol, 2% b-mercaptoethanol, 4% SDS, 0.13 M Tris-HCl, 0.05% bromo-phenol blue, pH 6.8), brought to 75°C, and heated for 10 min. This was designated the time zero sample. The remainder was incubated at 37°C for 2 min prior to being neutralized and heated in the same manner as the time zero sample.

- **Time Course of Degradation.** Pepsin concentrations for time course experiments were selected based on the results of the digestion experiments described above (see **Pepsin concentration digestion experiments**). For experiments with maize-expressed CryIA(b), 0.001X pepsin in SGF was selected. For experiments with native CryIA(b), 0.01X pepsin in SGF was used. Reactions were initiated by addition of 40 µl of sample to 360 µl of SGF containing the selected pepsin concentration. At time 0, and after 1, 2, 5, 10 and 30 min of incubation at 37°C, 50 µl aliquots were removed. A control was also analyzed in which 10 µl of sample and 90 µl of SGF prepared without pepsin were mixed and sampled at time zero and after 30 min of incubation at 37°C. Samples from all time points were immediately neutralized and heated as described above (see **Pepsin concentration digestion experiments**).**Western Blot Analysis of Maize CryIA(b) Digests.** A 30 µl aliquot was taken from each reaction sample collected during the maize CryIA(b) digestibility experiments and was subjected to SDS-PAGE on 10% polyacrylamide gels (Novex, San Diego, CA). The proteins were electroblotted onto Immobilon-P® membrane (Millipore, Bedford, MA) and developed using immunoaffinity purified rabbit antibodies specific for the insecticidal crystal proteins from *B.t.k.* strain HD-1 (0.125 mg antibody/ml blocking buffer containing 3% nonfat dry milk, 10 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween 20, pH 7.5). Goat anti-rabbit IgG linked to horseradish peroxidase (Bio-Rad, Hercules, CA), diluted 1:3000 in blocking buffer, was used to bind to the primary antibody and was detected using an ECL™ kit (Amersham, Arlington Heights, IL).

- **SDS-PAGE Analysis of Native CryIA(b) Digests.** SDS-PAGE was run on 30 µl aliquots of each reaction mix containing native CryIA(b) as described above (see **Western blot analysis of maize CryIA(b) digests**) for maize CryIA(b). After

staining with Coomassie blue, gels were examined for the presence of native CryIA(b) (approximately 128,000 mol. wt.) and degradation products.

D. Results

- **Pepsin Concentration Dependent Digestion.** In the presence of SGF at the standard pepsin concentration (1X), the maize CryIA(b) sample was completely degraded at time zero as determined by western blot analysis (Figure 1). The native CryIA(b) sample was nearly completely degraded after 2 min (Figure 2). Although the same amount of total protein was added to the SGF for both the maize and native CryIA(b) digests, a direct quantitative comparison of CryIA(b) degradation in the two digests is not possible because the CryIA(b) concentration of the native CryIA(b) sample was higher.

- After 2 min in SGF prepared with 0.001X the standard pepsin concentration, a significant amount of maize CryIA(b) had not been degraded. Similar results were obtained for the native CryIA(b) sample in SGF prepared with 0.01X pepsin. Therefore, these pepsin concentrations were selected for the time-course digestion experiments with each source of CryIA(b).

- **Time Course of Digestion.** Maize-expressed CryIA(b) was undetectable after 10 min using 0.001X pepsin in SGF (Figure 3). The proportion of lower molecular weight immunoreactive fragments seen at time zero was increased after one minute of incubation as the approximately 65,000 mol. wt. protein was degraded. In the presence of 0.01X pepsin in SGF, native CryIA(b) was undetectable after 5 min; the full-length (approximately 128,000 mol. wt.) CryIA(b) protein as well as intermediate (> approximately 56,000) molecular weight polypeptides visible at the initial sampling time appeared to be completely degraded (Figure 4).

E. Discussion

The results of this study indicate that maize-expressed CryIA(b) protein is readily degraded in a simulated mammalian gastric environment. At the standard pepsin concentration, the protein was degraded so quickly that no immunoreactive material could be detected by western blot analysis upon immediate sampling of the reaction mix. When pepsin was diluted to 0.001X its standard concentration in SGF, a time-course of maize CryIA(b) degradation was demonstrated; under these conditions where the proteolytic enzyme was significantly diluted, western blot analysis showed no immunoreactive CryIA(b) polypeptides after 10 min. These data suggest that maize-expressed CryIA(b) protein will be readily digested in the mammalian gastric environment as conventional dietary protein.

F. ACUTE ORAL TOXICITY STUDIES IN MICE OF CryIA(b) PROTEIN

1. Studies using native CryIA(b) protein

The test material used for this first study, conducted by Stillmeadow, Inc. (Sugar Land, TX), was a purified native CryIA(b) produced through fermentation of *B.t.k.* strain HD1-9. The CryIA(b) protein was purified according to procedures detailed in Chapter 3, Appendix 3C of this report. The CryIA(b) content of this material was determined to be at least 65% by weight. This endotoxin content is considerably higher than the typical ca. 10 - 15% endotoxin content of technical grade spray-dried fermentation materials used to

formulate *Bt* microbial preparations. The CryIA(b) preparation was prepared as a suspension in carboxymethylcellulose and administered to 5 male and 5 female mice in a single oral gavage dose of 5050 mg/kg. Since the CryIA(b) content of the test material was at least 65%, the actual amount of CryIA(b) protein dosed was at least 3283 mg/kg. This likely far exceeds the endotoxin doses that have typically been administered in acute testing programs of *Bt* microbial products. No mortality occurred during the 14-day study, and the only clinical sign observed was piloerection on Day 1. No meaningful effects on body weight gain were observed during the study, and no abnormalities were noted upon gross necropsy.

Contrasted with the trace levels of CryIA(b) protein detected in corn kernels, the single 3283 mg CryIA(b)/kg acute dose administered to the test mice far exceeds any potential lifetime exposures for mammalian species, including domestic animals, the highest corn grain consumers. For example, a hog consumes approx. 300 kg of corn grain from weaning until it reaches market weight. If it is assumed that CryIA(b) is present at the limit of quantification (5 ppb) in kernels, the lifetime consumption of CryIA(b) protein by a hog would be approximately 1.5 mg. In contrast, a 25-g mouse exposed during the toxicity study would have received 82 mg of CryIA(b) in a single dose.

2. Studies using truncated CryIA(b) protein

The test material used for this study, conducted by Stillmeadow, Inc. (Sugar Land, TX), was an enriched maize leaf protein preparation containing 0.07%, by weight, truncated CryIA(b) protein. The extract was derived from event 176 transformed maize leaf tissue, utilizing procedures described in Chapter 3, Appendix 3A of this report. The CryIA(b) preparation was prepared as a suspension in carboxymethylcellulose and administered to 5 male and 5 female mice in a single oral gavage dose of 5050 mg/kg (Test Group). The Control Group consisted of 5 male and 5 female mice that were administered an equivalent dose of a preparation from non-transformed maize tissue that was isolated in an identical manner to that used to prepare the CryIA(b) material. Within one day of treatment, one female in the Control Group and one female in the Test Group died. Although these deaths may have been treatment related, no incremental mortality was seen in the Test Group. There were no significant differences in clinical findings or body weight gain between the group receiving control maize protein and the group administered CryIA(b)-containing maize protein. No gross abnormalities were noted at necropsy among any of the mice surviving to study termination at 14 days. One additional death occurred on Day 2 (of a Bt maize treated male) and was attributed to a dosing injury; necropsy revealed a punctured esophagus. It cannot be ruled out that a maize protein component, present in both the control and Bt maize preparations, was responsible for the low incidence of mortality observed in this study. The acute oral LD_{50} for Bt maize leaf protein, as indicated by this study, is determined to be greater than 5050 mg/kg when administered to male and female mice.

The total amount of truncated CryIA(b) protein ingested by the mice in this study was substantially less than the amount of full-length CryIA(b) protein ingested in the first study cited above. However, if it is assumed that CryIA(b) is present at the limit of quantification (5 ppb) in kernels, then the mice in our study would have had to consume 17.6 kg, or 704x their body weight in corn kernels to have received the equivalent amount of CryIA(b) protein administered in this study.

3. Assessment of Mammalian Toxicity Data

The absence of toxicity in the acute oral mouse study coupled with the immediate degradation of the CryIA(b) protein in a simulated mammalian gastric environment indicates that the CryIA(b) protein as produced in *Bt* maize will be of no toxicological significance to mammalian species.

G. FOOD AND FEED PRODUCTS DERIVED FROM CORN PROCESSING

Based upon the current distribution of no. 2 yellow dent field corn grain in commerce, it is estimated that at least 60% of Ciba Seeds' Event 176 Bt corn grain will ultimately be used directly by US farmers as animal feed. However, up to 20% may find its way into various human food products and animal feed formulations, with the remaining 20% being exported (National Corn Growers Association, 1992). Many of the processes involved in producing these corn feeds and by-products significantly reduce total protein content below the 10% level found in grain, or significantly eliminate, degrade or denature the constituent proteins due to extremes of temperature and pressure. These same processes would also serve to significantly reduce or eliminate the trace levels of CryIA(b) (< 5 ppb) detected in kernels from Event 176. This is relevant to the overall safety assessment of *Bt* maize as the primary route of exposure to transgenic proteins from corn would be dietary.

1. Description of Milling Processes, Products and Applications

- **Dry Milling.** The primary products of the corn dry milling process are grits, cornmeal and corn flours. The largest food/feed applications of the products of dry milling are animal feed, followed by brewing, food uses, including breakfast cereals, mixes, baking, and snack and other foods.

  The various by-product streams resulting from corn dry milling processes are most frequently combined to produce a single product known as hominy feed, an important ingredient in animal feed. Protein content in dry-milled protein by-products ranges from a low of 8% for corn bran and hominy feed, to a high of 16% for corn germ, while standard meal contains 11% protein. Other processed corn products such as breakfast cereals, bread, tacos and chips typically contain 5 - 8% total protein by weight. Some by-products of corn processing have higher total protein content than the raw commodity. Corn gluten feed contains approximately 21% total protein by weight, while corn gluten meal contains approximately 70% total protein.

  Virtually all processing of whole corn and dry-milled fractions into finished products involves exposure to high temperatures. For example, corn grits are processed into corn flakes and are exposed to drying at 66°C and later to toasting at temperatures as high as 302°C. Other breakfast cereals and snacks require exposure to pressures of up to 200 psi and temperatures to 427°C. Typical commercial corn and tortilla chip processing involves cooking whole corn at 100°C followed by baking or frying. Extrusion cooking is a commonly used processing tool for food uses of dry milled corn. Heat may be generated throughout the process as a result of shear forces within the cooker or may be applied from external sources such as steam. The materials may be subjected to pressures of up to 1000 psi during processing.

  CryIA(b) protein undergoes irreversible denaturation and complete loss of insecticidal activity at elevated temperatures (80-100°C for 5 min). Dry milling process conditions

would denature any minimal CryIA(b) residues and eliminate any biological activity, regardless of the protein content of these by-products.

- **Wet Milling.** This process produces starch products, food sweeteners, corn gluten feed and corn gluten meal. Wet milling involves an initial soaking (steeping) in water under processing conditions, followed by milling and separation by screening, centrifugation and washing. The initial soaking typically exposes the kernels to 52°C for as long as 50 hr. It is during this process that the material is also exposed to SO_2 at a maximum concentration of 0.20%.

  After germs and fiber have been separated from the milled corn, only high-protein substances, gluten and corn soluble impurities remain with the starch slurry. High-protein gluten is then separated from the starch by centrifugation. After further centrifugation, filtering and drying, the resulting high protein gluten meal (>68%, solids basis) is sold for use in animal feed formulations. After washing, the starch fraction contains up to 0.35% total protein and 0.01% soluble protein. Depending on the intended end-use of the starch, subsequent drying of the product can expose it to temperatures ranging from 65 - 220°C.

  Further processing for food uses involves more extensive cooking prior to grounding. The starch used to produce corn sweeteners is about 99% pure and contains 0.25 - 0.35% total protein on a dry basis. Regular corn syrups and high-fructose corn syrup are among the nutritive sweeteners manufactured from wet-milled corn starch. Finished starch may also be fermented to produce ethanol. Soluble components extracted from the corn during steeping are routed to evaporators to increase solids, which are then mixed with corn fiber and processed into corn gluten feed. After exposure to temperatures as high as 700°C during the drying process, the finished product contains approximately 21% protein.

  Corn germs, another by-product of wet milling, are processed for their oil. After separation from the corn slurry, they are dewatered and dried. A solvent-based process, or pressure and heat to 120°C are applied to extract the oil. Most of the resulting corn oil is then further processed into frying or salad oils and margarine. Non-protein corn products such as high fructose corn syrup, corn oil and corn starch contain less than 1% total protein by weight; therefore it is highly unlikely that any CryIA(b) protein would be detectable in these products.

- **Animal Feeds.** In addition to whole or ground grain and cob, many wet- and dry-milled corn by-products are used for animal feed formulations. Gluten feed, gluten meal and hominy feed are among them, with protein contributions ranging from 10.6% for hominy to 61.2% for gluten meal. Silage and cobs supply the lowest amount of crude protein when included as animal feed, at approximately 3%. A broad range of corn products are used in formulating feed for swine, sheep, beef and dairy cattle, warmwater fish and shellfish.

  Corn is the chief grain fed to poultry. As with swine, both grain and various milled by-products are used in the formulation of poultry feed. Corn is fed to goats as ground cobs, whole or ground grain and silage. Ground corn grain can comprise as much as 88% of standard diet for sheep. Corn as a component of diet for fish and shellfish ranges from no use at all for shrimp or common carp, to approximately 35% for channel catfish.

The processes by which these products are produced involve high temperatures and/or other conditions which result in protein denaturation and in some cases, even cleavage of the peptide backbone (e.g., high temperature combined with highly pressurized conditions). As with the vast majority of characterized proteins the transgenic proteins in *Bt* grain would be expected to denature when exposed to temperatures above 100° C. As a result, it is very unlikely that any biologically active CryIA(b) protein would be detectable in processed food products. The same is true of many animal feed formulations. Ciba Seeds attempted to detect CryIA(b) after using event 176 grain as the sole corn source in formulating fish feed for toxicity testing. At a concentration of approximately 35% corn in the feed formulation, no CryIA(b) protein was detectable by ELISA, and the feed was not bioactive against European corn borer, the target pest. Moreover, this feed had been deliberately prepared with a minimum of heat and pressure, unlike many extruded or pelletted animal feeds, in an attempt to preserve as much protein integrity as possible.

Several factors combine to indicate that CryIA(b) residues will not occur in animal feeds, or will occur at undetectable levels well below the 5 ppb lower limit of quantification.

Conclusion

The transgenic proteins in Ciba Seeds' Event 176 Bt corn should pose virtually no risk to human or animal health in processed corn feed or food products due to:

- The relatively low expression level in the kernel (Chapter 4);
- The low total protein content in most corn products
- The extensive exposure to protein denaturing conditions during processing;
- The actual dilution of any one source of corn grain in a processing stream that mixes and uses many different corn varieties;
- The digestibility of the proteins in a simulated gastric environment (Chapter 8);
- The lack of mammalian toxicity of the CryIA(b) protein (Chapter 8)

H. References

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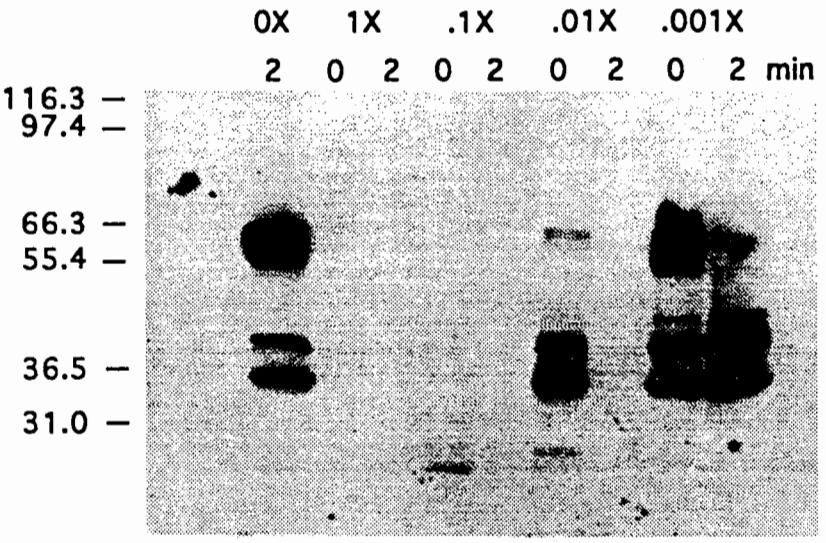

Figure 1. Digestion of maize CryIA(b) (approx. 65,000 mol. wt.) in simulated gastric fluid (SGF) prepared at various concentrations of pepsin.

Digestion reactions were prepared and maize CryIA(b) degradation was visualized via western blot analysis as described in Materials and Methods. SGF was used at the standard concentration of pepsin (1X), or prepared with 0.1X, 0.01X, and 0.001X the standard pepsin concentration. SGF prepared without pepsin was designated 0X. Each lane represents a sample that initially contained 15 mg of CryIA(b)-enriched maize leaf protein corresponding to 11 ng of maize CryIA(b) protein. Molecular weight standards (x 10^3) are indicated.

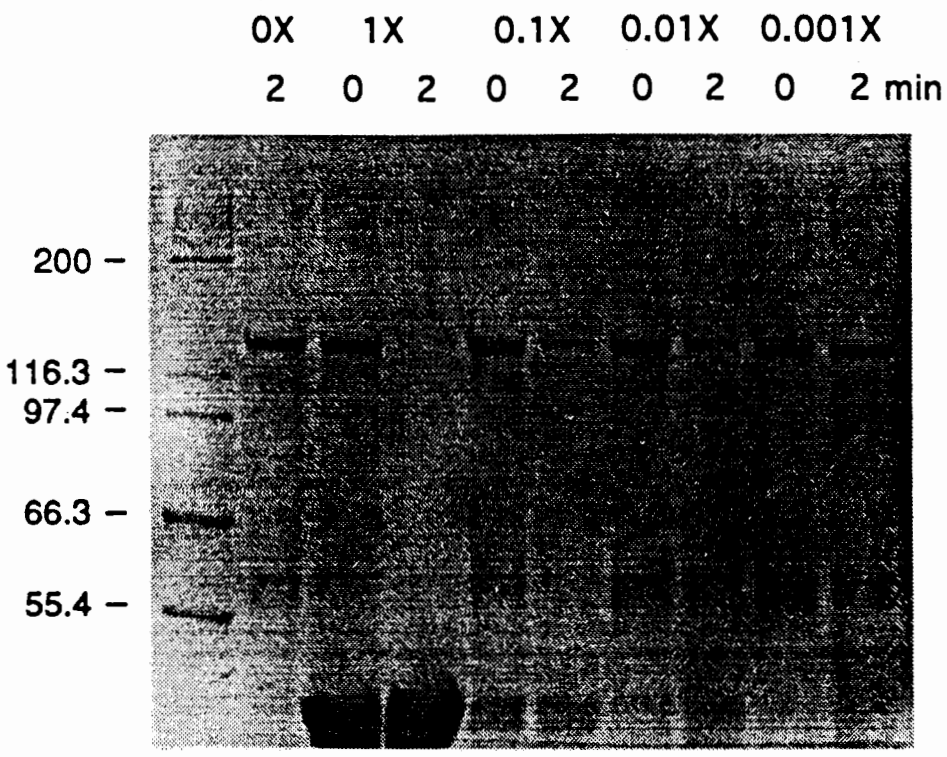

Figure 2. Digestion of native CryIA(b) (approx. 128,000 mol. wt.) in simulated gastric fluid (SGF) prepared at various concentrations of pepsin.

Digestion reactions were prepared and native CryIA(b) degradation was visualized via Coomassie blue staining as described in Materials and Methods. SGF was used at the standard concentration of pepsin (1X) or prepared with 0.1X, 0.01X, and 0.001X the standard pepsin concentration. SGF prepared without pepsin was designated 0X. Each lane represents a sample that initially contained 15 mg of test protein, corresponding to approximately 7 mg of full length native CryIA(b) from *Bacillus thuringiensis* subsp. *kurstaki*. Molecular weight standards (x 10^{-3}) are indicated.

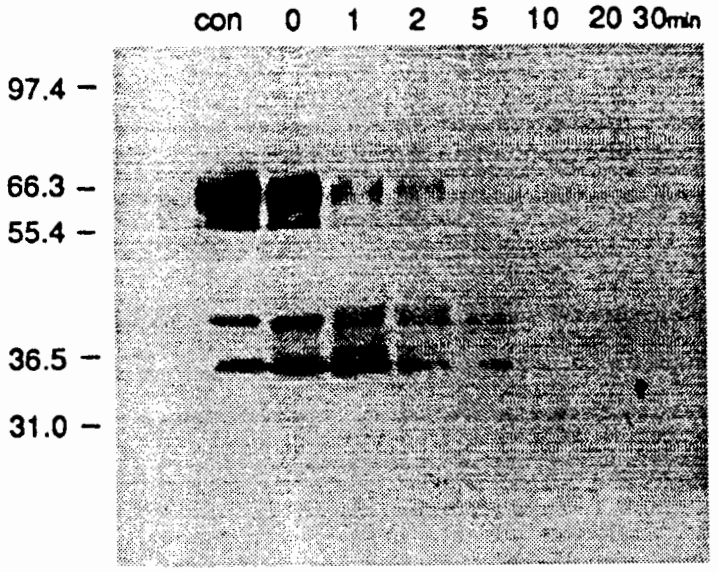

Figure 3. Time course of digestion of maize CryIA(b) (approx. 65,000 mol. wt.) in 0.001X pepsin in simulated gastric fluid (SGF).

Western blot analysis was used to visualize proteolytic degradation of maize-expressed CryIA(b) and immunoreactive CryIA(b) fragments. Samples were incubated for the indicated times in 0.001X pepsin in SGF as described in Materials and Methods. The control was incubated in SGF minus pepsin for 30 min at 37°C. Each lane represents a sample that initially contained 15 mg of CryIA(b)-enriched maize leaf protein, corresponding to approximately 11 ng of maize CryIA(b) protein. Molecular weight standards (x 10^3) are indicated.

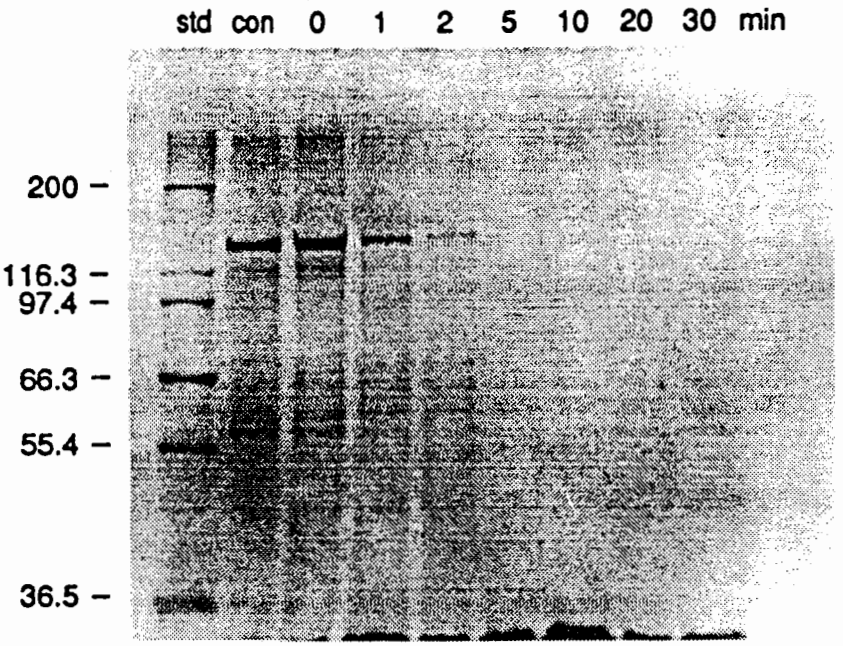

Figure 4. Time course of digestion of native CryIA(b) (approx. 128,000 mol. wt.) in 0.01X pepsin in simulated gastric fluid (SGF).

Native CryIA(b) degradation as a function of incubation time in 0.01X pepsin in SGF was visualized by Coomassie blue staining following electrophoresis on a 10% polyacrylamide SDS gel. Digestion reactions and sample handling were as described in Materials and Methods. The control was incubated in SGF minus pepsin for 30 min at 37°C. Each lane represents a sample that initially contained 15 mg of test material corresponding to 7 mg full length native CryIA(b) from *Bacillus thuringiensis* subsp. *kurstaki*. Molecular weight standards (x 10^{-3}) are indicated.

CHAPTER 9

Compositional Analysis of *Bt* Maize

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9. COMPOSITIONAL ANALYSIS of *Bt* MAIZE

A. Proximates, Carotenoid, Amino Acid, and Fatty Acid Determinations

Summary

A comparative study of the major components of event 176 derived maize and non-transgenic controls was performed. The composition of kernels was examined in two inbred lines and four hybrids representing early, middle, and late maturity groups. Proximates (ash, fat, fiber, moisture, protein, starch), carotenoid levels (xanthophylls and ß-carotene), amino acid composition, and fatty acid profiles were determined. Sporadic differences between the *Bt* and control maize kernels were observed for some of the components, but no pattern emerged that would indicate that these differences were attributable to the transformation process or the expression of the transformed genes in the *Bt* plants. The findings of this study indicate that the composition of event 176 derived maize kernels is indistinguishable from their non-transformed counterparts.

Materials and Methods

- **Tissue Description**

  Kernels analyzed were derived from two inbred lines and four hybrids:

  1. Kernels from the transgenic homozygous *Bt* maize inbred CG00526-176 and its isogenic control CG00526, grown at the Molokai, HI field station, were shipped to Bloomington, IL for processing and transported to the Ciba facility at Research Triangle Park, NC. Kernels were stored under standard seed storage conditions (10 ± 1°C and 50 ± 10% relative humidity).

  2. Kernels from three hybrid crosses [CG00637 x CG00526-176 (early maturity); CG00684 x CG00526-176 (middle maturity); CG00554 x CG00526-176 (late maturity)] and their corresponding controls were also grown at the Molokai, HI field station and shipped as above.

  3. Kernels from one *Bt* inbred line (CG00615-176; field grown, Lombez, France.) and the corresponding control line (CG00615; field grown; Lombez, France) as well as kernels from two hybrids (CG00635 x CG00615-176; CG00635 x CG00615) produced in a greenhouse in Dijon, France, were shipped directly to the facility in Research Triangle Park, NC.

  All US interstate movement and importations of seed were conducted in full compliance with USDA regulations.

- **Tissue Processing**

  For the inbreds and hybrids grown in Hawaii, five random 100-g samples of kernels of each genotype were taken from a pooled sample representing multiple plants. These were used for all analyses except fatty acid determinations. For the inbreds and hybrids grown in France, two 12.5-g samples of kernels were taken from a pooled sample representing multiple plants. Fatty acid and amino acid analyses were not conducted on the French samples.

Fatty acid analysis of embryos necessitated their isolation from the kernel. Seed was soaked in 20% bleach for 60 min, rinsed (3X) for 10 min in sterile distilled water, and soaked overnight in sterile distilled water. Embryos were excised, immediately frozen on dry ice, and stored at -80° C until analyzed.

- **Analyses**

All tissue samples were shipped on dry ice to Southern Testing and Research Laboratories, Inc., Wilson, NC for analysis. For the inbreds and hybrids grown in Hawaii, analyses were performed once on each of the five samples. For the material from France, analyses were performed twice on each of the two replicate kernel samples. The following methods were employed:

Test	Method[1]
ash	AOAC 942.05
fat	AOAC 920.39
crude fiber	AOAC 962.09
moisture	AOAC 930.15
protein	AOAC 990.03
starch	AACC 76-11
carotenoids	AOAC 970.64, modified to use HPLC
amino acids	method of Hagen, *et al* (1989)
fatty acids	extraction by AOAC 983.23; derivatization and quantitation by AOCS Ce 1-62, modified to use capillary GC

For each analyzed parameter, means and standard deviations were calculated. Transgenic inbreds and hybrids were compared to their corresponding nontransgenic controls by *t*-tests (for unequal variances). Significance was judged at the level of $p = 0.05$.

Results

Comparison of proximate values from *Bt* lines and their isogenic controls revealed little differences (Table 1; Figures 1 - 6). No statistically significant differences were found for any lines with respect to their ash or fiber content. There were some isolated differences in protein, starch, and fat content between some of the *Bt* lines and their isogenic controls, but these differences are minimal, within the established range for maize (Watson, 1987), and considered to be biologically and nutritionally insignificant. No pattern emerged that would indicate that these differences were due to the presence of the transgene.

Xanthophyll levels were quite variable between the different genotypic maize backgrounds, but no statistically significant differences were observed between any *Bt* inbreds or hybrids and their corresponding controls (Table 2; Figure 7). Similarly, ß-carotene levels varied by genotypic background. One *Bt* inbred and its control were significantly different in ß-carotene levels, while the remaining inbreds and hybrids demonstrated no differences. The observed differences in carotenoid levels between the different maize genotypic backgrounds may be in part related to differences in the length

[1]AOAC = Association of Official Analytical Chemists; AACC = American Assoc. of Cereal Chemists; AOCS = American Oil Chemists Society

of time the grain was stored. Carotenoid levels have been shown to decrease with time in storage (Watson, 1962).

Although sporadic statistically significant differences in the relative proportion of one or more amino acids were observed in all the *Bt* maize inbreds and hybrids, the differences were small and did not alter the overall amino acid profile (Table 3; Figures 8 - 11)

The major fatty acids present in maize embryos exhibited the same relative levels in transgenic and non-transgenic lines (Table 4, Figure 12). No statistically significant differences were observed.

B. Analysis of DIMBOA levels in *Bt* Maize

Introduction

Hydroxamic acids are natural constituents of some plant species and have been implicated to have a role in resistance of cereals to certain insects, fungi, and bacteria (Niemeyer, 1988). In particular, a class of hydroxamic acids, 4-hydroxy-1,4-benzoxazin-3-ones, have been studied more extensively and the evidence supporting their role in warding off pests is substantial.

DIMBOA (2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one) is the predominant hydroxamic acid present in maize tissue (Klun *et al*, 1967). DIMBOA production is genotype specific and its synthesis temporally controlled (Klun and Brindley, 1966; Dunn *et al*, 1981). Greater quantities are generally found in young maize seedlings (Klun and Robinson, 1969), tending to be concentrated in the vascular tissue of the distal portions of leaves (Niemeyer, 1988).

Hydroxamic acids, such as DIMBOA, exist as glucosides *in vivo*, but undergo rapid enzymatic hydrolysis during the extraction process. DIMBOA further degrades spontaneously (non-enzymatically) to the stable compound MBOA (Brendenberg *et al*, 1962; Woodward *et al*, 1978)). Laboratory methods have been developed which promote the efficient conversion of the unstable DIMBOA molecule to MBOA, thus enabling quantification of MBOA to be a direct reflection of the amount of DIMBOA present in the plant tissue prior to extraction.

In this study we examined whether maize plants derived from event 176 have altered levels of DIMBOA as a result of the transformation process or due to the expression of foreign genes. DIMBOA levels were determined in *Bt* maize plants derived from event 176 and compared to levels in isogenic control plants grown simultaneously under greenhouse conditions. No significant differences in DIMBOA levels were detected between transgenic and control plants.

Materials and Methods

- **Source of Plant Material.** *Bt* maize plants, CG00526-176, homozygous for the transgenes[1], and isogenic nontransformed controls were planted on 9 Feb 94 and grown simultaneously under greenhouse conditions without the application of

[1]Homozygosity of the *Bt* maize families was established by the observation that progeny from self-fertilizations were uniformly tolerant to ECB. (Self-fertilization of a homozygous *Bt+/Bt+* parent yields progeny that are uniformly *Bt+/Bt+*.)

insecticides or fungicides. The distal half of the first expanded leaf of each plant (4 leaf stage) was harvested on 8 Mar 94 and stored at -80°C until extracted and analyzed.

- **Extraction of Leaf Material.** Leaf material (0.2 g) was ground in a mortar and pestle in the presence of liquid nitrogen. The powdered tissue was suspended 1:2 (w:v) in 10 mM sodium phosphate, pH 7.0, and incubated for 1 h at room temperature to allow enzymatic hydrolysis of the DIMBOA glucoside (Sahi *et al.*, 1990). The samples were then heated for 1 h at 100°C to facilitate the conversion of DIMBOA to MBOA. Particulates were removed by centrifugation for 10 min at 5000 x g and the supernatant was extracted three times with an equal volume of ethyl acetate. The ethyl acetate fractions were pooled for each sample, and removed by evaporation under nitrogen. The residue was dissolved in methanol and used for MBOA analysis by HPLC.

- **HPLC Fractionation and MBOA Quantitation.** Extracts prepared as described above were filtered through an Acrodisc® (Gelman, Ann Arbor, MI) prior to being fractionated using an HP1090 Liquid Chromatograph (Hewlett Packard, Avondale, PA) equipped with a Velosep™ reverse phase C-18 column (3µm, 3.2 x 100 mm) (Applied Biosystems, Foster City, CA). Solvent conditions were as follows: A = 50 mM sodium phosphate, pH 7.0, B = 100% acetonitrile.

Time (min)	%A	%B
2	100	0
20	50	50
22	5	95
28	5	95
29	100	0

Solvents were changed by a linear gradient from indicated concentrations of A to B over the time indicated. Flow rate was maintained at 1 ml/min and output was monitored at 280, 262 and 220 nm.

MBOA eluted at approximately 14 min. MBOA was quantitated by comparison with signal intensity at 280 nm generated by an MBOA standard (Sigma, St. Louis, MO).

Results

DIMBOA levels (as determined after conversion to MBOA) were not significantly different between the *Bt* maize plants and the isogenic controls (Table 5). The mean value obtained for the *Bt* maize plants was 0.84 ± 0.36 mg MBOA/g fresh wt. as compared to that for the isogenic control of 0.86 ± 0.36. This result indicates that the process of transformation or foreign gene expression did not alter DIMBOA levels in plants derived from event 176. This result is further evidence that event 176 derived maize plants are indistinguishable from their non-transformed counterparts except for their resistance to lepidopteran pests and glufosinate tolerance.

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TABLE 1: PROXIMATE ANALYSES OF KERNELS FROM CONTROL AND *BT* MAIZE

Maize Genotype[1]	N[2]	Mean Content (% dry weight of sample) ± SD (range)					
		Ash	Fiber	Fat	Moisture[3]	Protein	Starch
526	5	1.44 ± 0.03 (1.38-1.46)	1.95 ± 0.08 (1.85-2.05)	4.74 ± 0.80 (3.84-5.65)	11.94 ± 0.44 (11.28-12.49)	12.21 ± 0.43 (11.60-12.74)	65.85 ± 4.17 (60.86-72.08)
526-176	5	1.41 ± 0.04 (1.35-1.45)	1.86 ± 0.13 (1.69-2.03)	4.07 ± 0.80 (3.30-5.38)	12.38 ± 0.34 (12.02-12.82)	11.71 ± 0.35 (11.23-12.05)	69.95 ± 0.88 (68.69-70.84)
554 x 526	5	1.30 ± 0.05 (1.26-1.36)	1.50 ± 0.13 (1.30-1.65)	2.55 ± 1.14 (0.95-3.90)	9.64 ± 0.40 (9.29-10.27)	11.96 ± 0.35 (11.43-12.33)	68.29 ± 10.06 (57.28-79.51)
554 x 526-176	5	1.27 ± 0.03 (1.22-1.30)	1.41 ± 0.12 (1.20-1.50)	4.21 ± 0.79* (3.35-5.28)	12.23 ± 0.30* (11.98-12.72)	10.88 ± 0.17* (10.70-11.11)	72.19 ± 2.56 (68.25-74.64)
637 x 526	5	1.63 ± 0.25 (1.28-1.90)	1.97 ± 0.10 (1.90-2.14)	4.07 ± 1.12 (2.64-5.08)	12.17 ± 0.49 (11.55-12.72)	12.13 ± 0.48 (11.53-12.72)	66.84 ± 2.97 (63.88-71.09)
637 x 526-176	5	1.68 ± 0.23 (1.31-1.90)	1.77 ± 0.32 (1.20-2.00)	3.49 ± 1.62 (1.01-5.54)	10.24 ± 1.88 (7.87-11.97)	13.62 ± 0.48* (13.14-14.14)	68.85 ± 2.29 (66.81-72.06)
684 x 526	5	1.73 ± 0.16 (1.45-1.84)	1.56 ± 0.38 (0.90-1.80)	3.66 ± 0.96 (2.35-5.04)	12.14 ± 0.28 (11.93-12.46)	12.85 ± 0.39 (12.51-13.48)	58.23 ± 7.19 (50.37-67.51)
684 x 526-176	5	1.63 ± 0.16 (1.36-1.74)	1.61 ± 0.16 (1.45-1.80)	2.04 ± 0.60* (1.45-2.89)	9.01 ± 1.27* (6.97-10.30)	13.32 ± 0.37 (12.85-13.87)	68.07 ± 3.01* (64.54-71.82)
615	2	1.73 ± 0.21 (1.58-1.87)	1.84 ± 0.08 (1.78-1.90)	4.67 ± 0.59 (4.25-5.08)	10.82 ± 0.26 (10.63-11.00)	10.07 ± 0.15 (9.96-10.17)	63.16 ± 0.93 (62.50-63.82)
615-176	2	1.82 ± 0.01 (1.81-1.83)	1.70 ± 0.22 (1.54-1.85)	4.34 ± 0.13 (4.24-4.43)	12.38 ± 0.04 (12.35-12.41)	11.79 ± 0.07* (11.74-11.84)	59.14 ± 0.98 (58.45-59.83)
635 x 615	2	1.93 ± 0.08 (1.87-1.99)	1.74 ± 0.06 (1.69-1.78)	4.14 ± 0.10 (4.07-4.21)	13.22 ± 0.27 (13.03-13.41)	11.17 ± 0.62 (10.73-11.60)	61.51 ± 0.75 (60.98-62.04)
635 x 615-176	2	1.81 ± 0.01 (1.80-1.82)	1.92 ± 0.23 (1.75-2.08)	4.05 ± 0.21 (3.90-4.19)	12.06 ± 0.10 (11.99-12.13)	11.38 ± 0.33 (11.14-11.61)	61.04 ± 1.82 (59.75-62.32)

[1] Abbreviations: 526 = CG00526 inbred; 554 x 526 = CG00554 x CG00526 hybrid; 637 x 526 = CG00637 x CG00526 hybrid; 684 x 526 = CG00684 x CG00526 hybrid; 615 = CG00615 inbred; 635 x 615 = CG00635 x CG00526 hybrid. "CG" numbers designate proprietary Ciba Seeds lines. The suffix "-176" indicates the transgenic *Bt* line/hybrid.

[2] Number of replicate samples analyzed from a pooled sample of kernels representing multiple plants. Where N = 5, samples were analyzed once. Where N = 2, each sample was analyzed twice.

[3] Moisture is given as percent of sample weight (prior to drying)

* Significantly different from the corresponding control mean ($p \leq 0.05$)

TABLE 2: COMPARISON OF CAROTENOID LEVELS IN CONTROL AND *BT* MAIZE KERNELS

Maize Genotype[1]	N[2]	Mean ± SD (µg/100 g of sample) (range) Xanthophylls	β-carotene
CG00526	5	323.6 ± 112.8 (231.3-512.3)	15.43 ± 1.18 (14.38-17.17)
CG00526-176	5	378.8 ± 37.5 (325.8-416.6)	17.38 ± 0.33* (17.03-17.86)
CG00554 x CG00526	5	377.1 ± 116.6 (216.7-541.1)	15.01 ± 0.96 (13.84-16.22)
CG00554 x CG00526-176	5	371.8 ± 50.0 (301.5-437.8)	15.20 ± 2.90 (11.94-19.66)
CG00637 x CG00526	5	284.7 ± 51.4 (212.8-350.9)	4.41 ± 4.04 (1.82-11.58)
CG00637 x CG00526-176	5	180.3 ± 86.2 (113.2-331.1)	3.18 ± 1.24 (2.36-5.38)
CG00684 x CG00526	5	237.9 ± 103.0 (132.7-353.4)	3.73 ± 0.80) (2.92-4.65)
CG00684 x CG00526-176	5	152.1 ± 40.8 (99.4-208.6)	2.80 ± 0.33 (2.39-3.13)
CG00615	2	3086.2 ± 67.7 (3038.3,3134.0)	60.92 ± 0.13 (60.83,61.01)
CG00615-176	2	2918.8 ± 314.5 (2696.4-3141.2)	47.88 ± 2.16 (46.35-49.41)
CG00635 x CG00615	2	1808.7 ± 126.3 (1719.4-1898.1)	41.43 ± 1.76 (40.18-42.67)
CG00635 x CG00615-176	2	1532.4 ± 113.0 (1452.5-1612.3)	29.25 ± 3.75 (26.59-31.90)

[1] "CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* inbred or hybrid.

[2] Number of replicate samples analyzed from a pooled sample of kernels representing multiple plants. Where N = 5, samples were analyzed once. Where N = 2, each sample was analyzed twice.

* Significantly different from control mean ($p \leq 0.05$)

TABLE 3: AMINO ACID COMPOSITION OF KERNELS FROM *BT* AND CONTROL MAIZE

Genotype[1]	Mean[2] Amino Acid Composition (% total protein) ± SD															
	Glu	Leu	Pro	Ala	Asp	Phe	Ser	Val	Arg	Gly	Thr	Tyr	Ile	Lys	His	Met
526	15.74 ± 0.63	10.69 ± 0.57	7.49 ± 0.22	6.29 ± 0.24	5.67 ± 0.32	4.75 ± 1.04	3.92 ± 0.16	3.81 ± 0.20	3.46 ± 0.28	2.96 ± 0.14	2.95 ± 0.15	2.94 ± 0.46	2.88 ± 0.33	2.34 ± 0.28	2.25 ± 0.10	1.92 ± 0.10
526-176	15.32 ± 1.08	11.08 ± 0.42	8.50* ± 0.41	6.47 ± 0.24	5.07 ± 0.47	3.87 ± 0.32	4.03 ± 0.24	3.70 ± 0.19	3.54 ± 0.21	3.30* ± 0.16	3.09 ± 0.12	2.92 ± 0.20	2.59 ± 0.08	2.48 ± 0.29	2.23 ± 0.11	1.76 ± 0.15
554 x 526	15.90 ± 0.45	10.82 ± 0.35	7.23 ± 0.23	6.77 ± 0.32	5.60 ± 0.18	4.03 ± 0.18	4.02 ± 0.14	3.66 ± 0.15	3.84 ± 0.20	3.00 ± 0.08	2.84 ± 0.05	2.85 ± 0.17	2.69 ± 0.05	2.36 ± 0.21	2.05 ± 0.05	1.70 ± 0.13
554 x 526-176	16.72 ± 0.68	11.60* ± 0.48	7.76 ± 0.30	6.82 ± 0.26	5.55 ± 0.25	4.93* ± 0.44	4.16 ± 0.15	3.87 ± 0.13	3.88 ± 0.18	3.14 ± 0.09	3.06* ± 0.16	2.84 ± 0.14	2.93* ± 0.13	2.55 ± 0.16	2.20* ± 0.09	1.72 ± 0.05
637 x 526	15.88 ± 1.15	10.66 ± 0.79	7.52 ± 0.51	6.43 ± 0.49	6.07 ± 0.42	5.20 ± 0.42	4.11 ± 0.27	3.83 ± 0.20	3.84 ± 0.26	3.25 ± 0.16	3.20 ± 0.13	3.06 ± 0.17	2.78 ± 0.17	2.99 ± 0.11	2.19 ± 0.15	1.51 ± 0.09
637 x 526-176	15.83 ± 0.69	10.84 ± 0.41	7.60 ± 0.46	6.12 ± 0.29	5.52 ± 0.32	4.82 ± 0.44	3.97 ± 0.17	3.50 ± 0.15	3.09* ± 0.19	3.05 ± 0.19	3.21 ± 0.18	2.87 ± 0.08	2.66 ± 0.19	2.20* ± 0.21	1.91* ± 0.09	1.35* ± 0.08
684 x 526	16.34 ± 1.15	11.34 ± 0.89	7.96 ± 0.78	6.25 ± 0.35	5.20 ± 0.31	5.48 ± 0.43	4.05 ± 0.28	3.41 ± 0.25	3.03 ± 0.26	2.86 ± 0.13	3.07 ± 0.24	3.23 ± 0.15	2.53 ± 0.34	1.89 ± 0.36	1.88 ± 0.16	1.52 ± 0.09
684 x 526-176	16.83 ± 0.88	12.02 ± 0.75	7.84 ± 0.42	6.54 ± 0.36	5.02 ± 0.28	5.96 ± 0.18	4.00 ± 0.18	3.27 ± 0.18	2.71 ± 0.22	2.70 ± 0.08	2.92 ± 0.16	3.35 ± 0.18	2.55 ± 0.15	1.45 ± 0.13	1.77 ± 0.08	1.37* ± 0.07

[1] Proprietary Ciba Seeds lines abbreviated as: 526 = CG00526; 554 x 526 = CG00554 x CG00526; 637 x 526 = CG00637 x CG00526; 684 x 526 = CG00684 x CG00526. The suffix "-176" indicates the transgenic *Bt* maize parent derived from Event 176. CG00526-176 is an inbred line that is homozygous for the transgenes.

[2] N = 5 replicate samples from a pooled sample representing multiple plants. Each sample was analyzed once.

* Significantly different from the corresponding control mean ($p \leq 0.05$).

TABLE 4: COMPARISON OF THE MAJOR FATTY ACIDS IN KERNELS FROM CONTROL AND *BT* MAIZE

			Content (% of total fatty acid)			
			Nontransgenic		Transgenic	
Maize Line[1]	Fatty Acid	N[2]	Mean ± SD	(range)	Mean ± SD	(range)
CG00526	16:0	5	12.71 ± 0.98	(11.77-14.33)	12.24 ± 0.57	(11.67-12.94)
	18:0	5	2.39 ± 0.58	(1.90-3.34)	2.20 ± 0.21	(1.95-2.48)
	18:1	5	27.09 ± 1.22	(24.45-28.34)	27.90 ± 0.74	(27.03-28.66)
	18:2	5	55.08 ± 3.45	(50.46-59.47)	55.38 ± 2.35	(52.13-58.08)
	18:3	5	0.73 ± 0.15	(0.52-0.83)	0.81 ± 0.08	(0.70-0.89)
CG00554 x CG00526	16:0	5	14.26 ± 0.61	(13.42-15.10)	13.77 ± 0.98	(12.92-15.32)
	18:0	5	2.26 ± 0.16	(2.03-2.42)	2.30 ± 0.17	(2.08-2.55)
	18:1	5	25.76 ± 0.34	(25.39-26.19)	25.69 ± 0.74	(24.86-26.86)
	18:2	5	54.78 ± 1.98	(52.36-57.36)	55.25 ± 2.73	(50.73-57.92)
	18:3	5	0.81 ± 0.04	(0.76-0.86)	0.88 ± 0.10	(0.77-0.97)
CG00637 x CG00526	16:0	5	12.92 ± 2.11	(11.41-16.09)	12.72 ± 1.14	(11.56-14.45)
	18:0	5	2.13 ± 0.41	(1.75-2.76)	2.16 ± 0.16	(2.00-2.36)
	18:1	5	30.04 ± 1.14	(28.57-31.18)	29.40 ± 0.93	(28.27-30.49)
	18:2	5	50.16 ± 6.92	(40.57-56.45)	51.12 ± 3.65	(45.37-54.48)
	18:3	5	0.84 ± 0.14	(0.61-0.96)	0.81 ± 0.09	(0.71-0.90)
CG00684 x CG00526	16:0	5	13.93 ± 1.65	(12.49-15.92)	14.17 ± 1.69	(12.60-16.23)
	18:0	5	2.15 ± 0.41	(1.79-2.65)	2.54 ± 0.50	(2.19-3.33)
	18:1	5	24.54 ± 1.18	(23.22-26.29)	24.39 ± 0.54	(23.98-25.26)
	18:2	5	55.86 ± 5.41	(49.74-60.71)	54.80 ± 4.64	(46.86-57.96)
	18:3	5	0.86 ± 0.13	(0.72-1.01)	0.89 ± 0.15	(0.64-1.02)

[1] "CG" numbers designate proprietary Ciba Seeds inbred lines. The suffix "-176" indicates the transgenic *Bt* inbred or hybrid.

[2] Number of replicate samples analyzed from a pooled sample of kernels representing multiple plants. Each sample was analyzed once.

TABLE 5: Levels of MBOA in *Bt* Maize and Nontransgenic Controls

Maize Line	Plant #	µg MBOA/g Fresh wt.
CG00526-176 (homozygous, transgenic line)	1	0.53
	2	0.71
	3	0.68
	4	1.56
	5	0.57
	6	1.38
	7	0.90
	8	0.71
	9	0.97
	10	0.35
	mean ± S.D.*	0.84 ± 0.36
CG00526 (control line)	1	0.53
	2	0.71
	3	0.94
	4	1.09
	5	0.61
	6	0.80
	7	0.76
	8	0.71
	9	0.57
	10	1.90
	mean ± S.D.	0.86 ± 0.38

*S.D. = Standard deviation

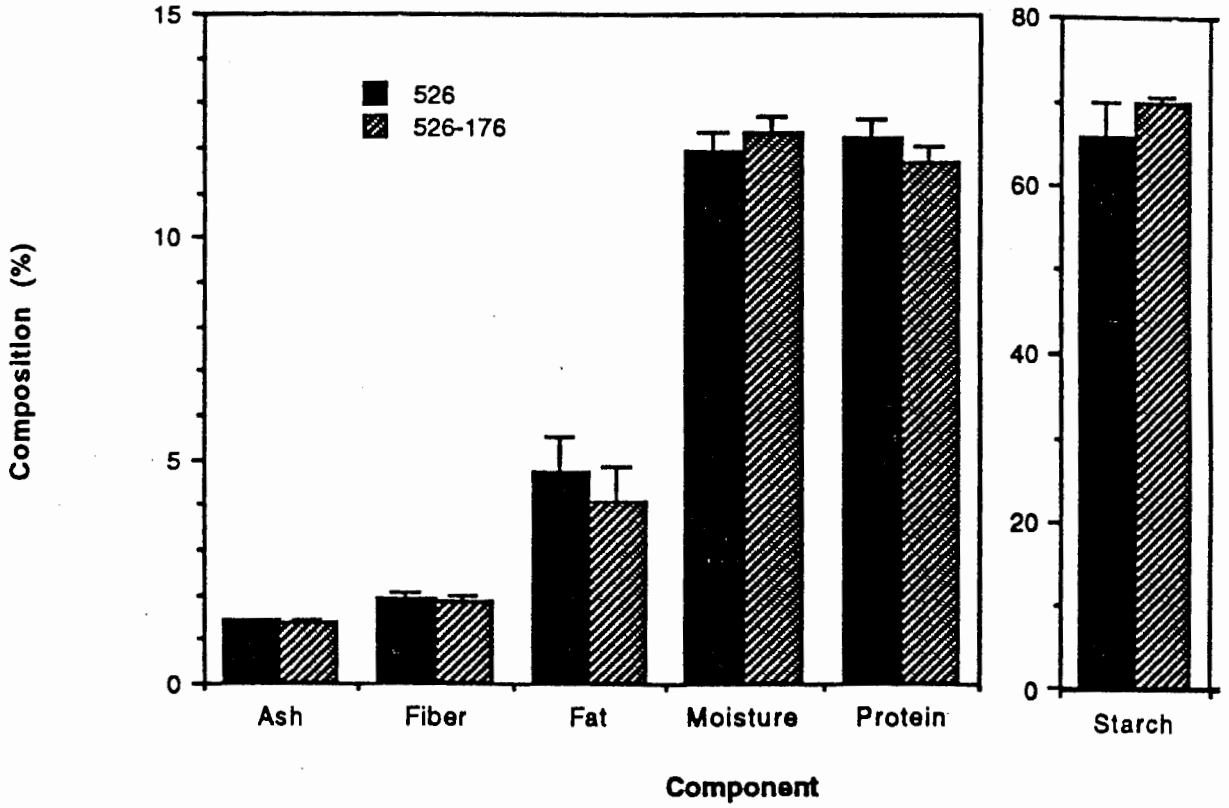

Figure 1. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize inbred CG00526-176 (hatched bars) and the corresponding nontransgenic inbred CG00526 (solid bars). Standard deviations are indicated.

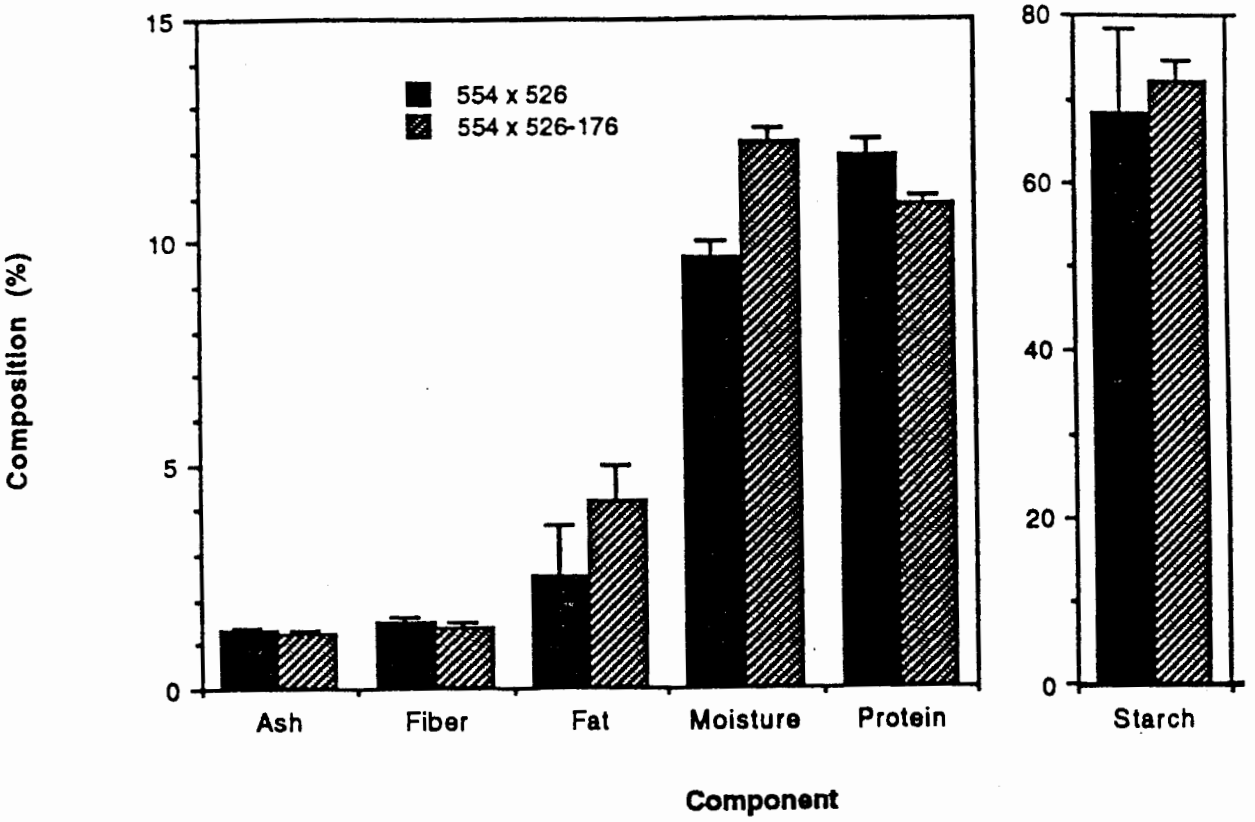

Figure 2. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize hybrid CG00554 x CG00526-176 (hatched bars) and the corresponding nontransgenic hybrid CG00554 x CG00526 (solid bars). Standard deviations are indicated.

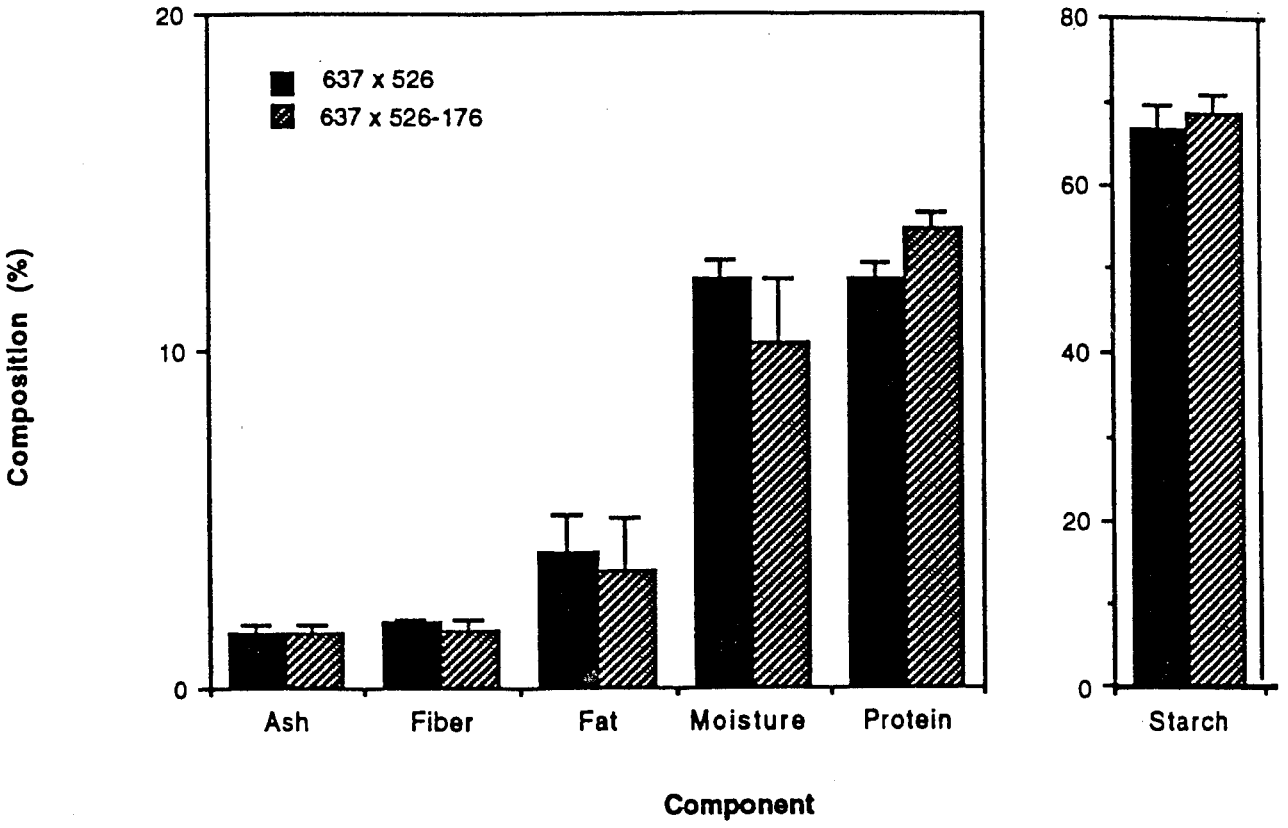

Figure 3. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize hybrid CG00637 x CG00526-176 (hatched bars) and the corresponding nontransgenic hybrid CG00637 x CG00526 (solid bars). Standard deviations are indicated.

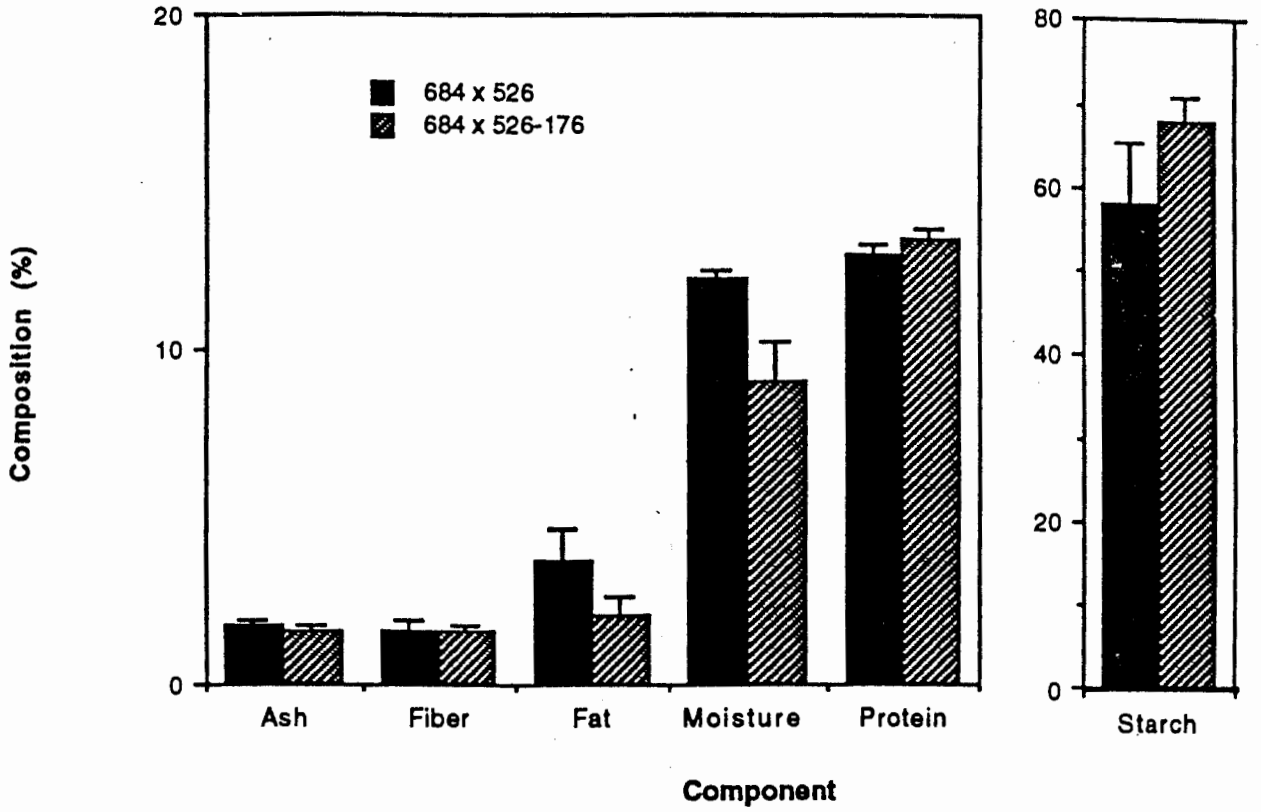

Figure 4. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize hybrid CG00684-176 x CG00526-176 (hatched bars) and the corresponding nontransgenic hybrid CG00684 x CG00526 (solid bars). Standard deviations are indicated.

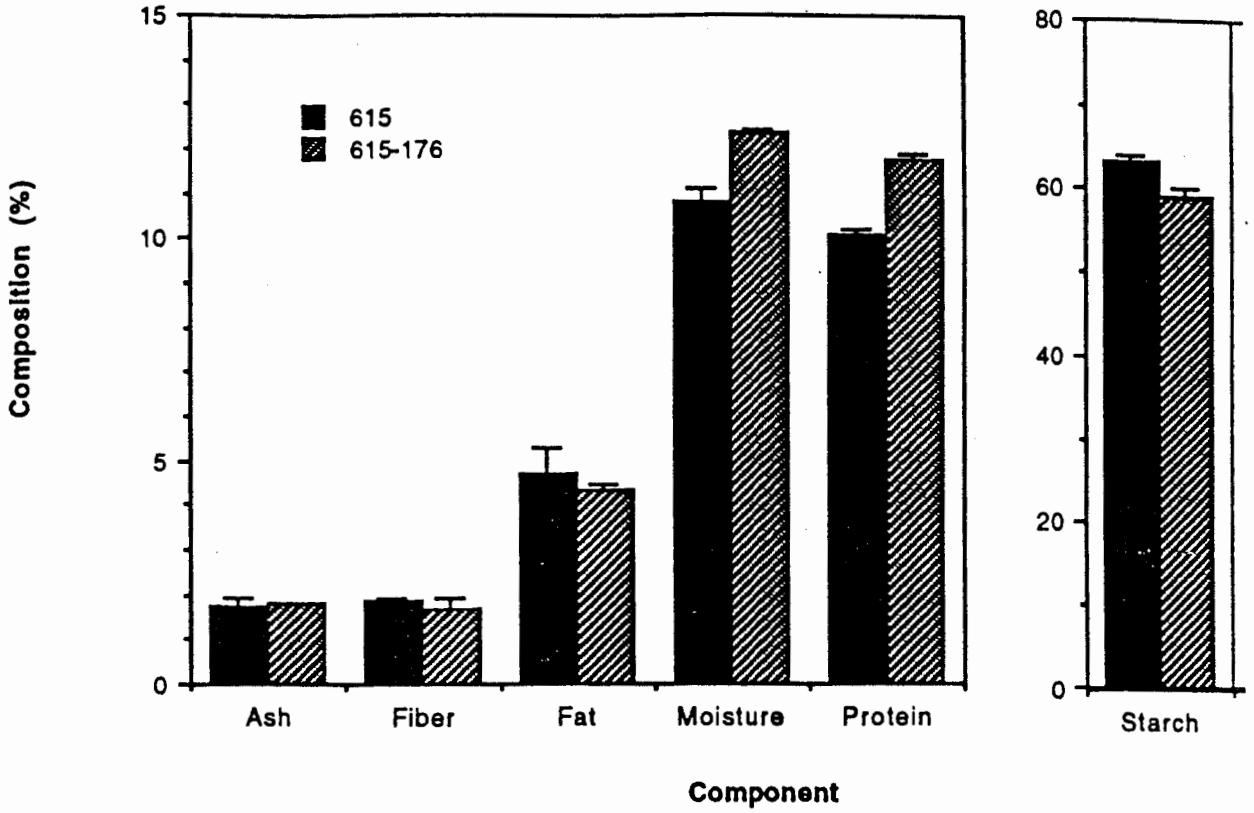

Figure 5. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize inbred CG00615-176 (hatched bars) and the corresponding nontransgenic inbred CG00615 (solid bars). Standard deviations are indicated.

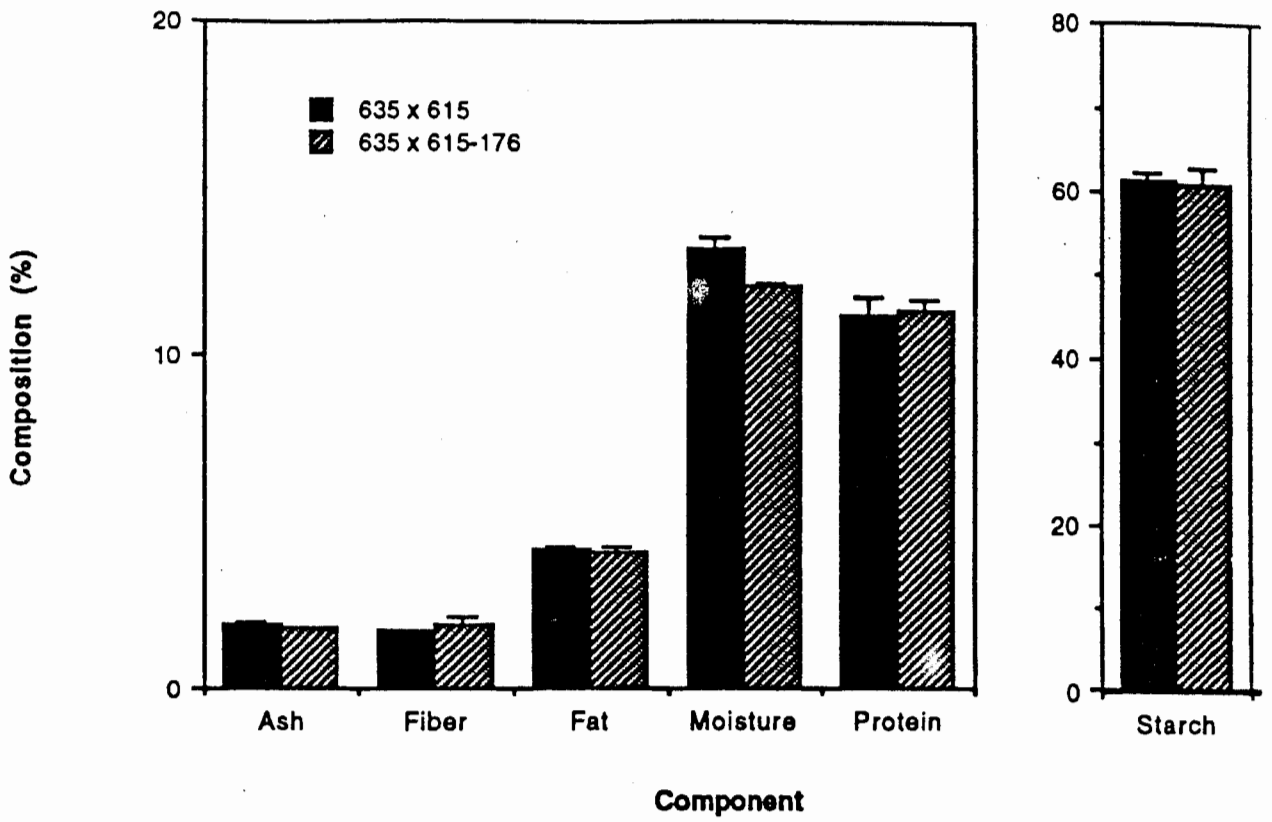

Figure 6. Graphic representation of mean proximate analyses values given in Table 1 for kernels of *Bt* maize hybrid CG00635 x CG00615-176 (hatched bars) and the corresponding nontransgenic hybrid CG00635 x CG00615 (solid bars). Standard deviations are indicated.

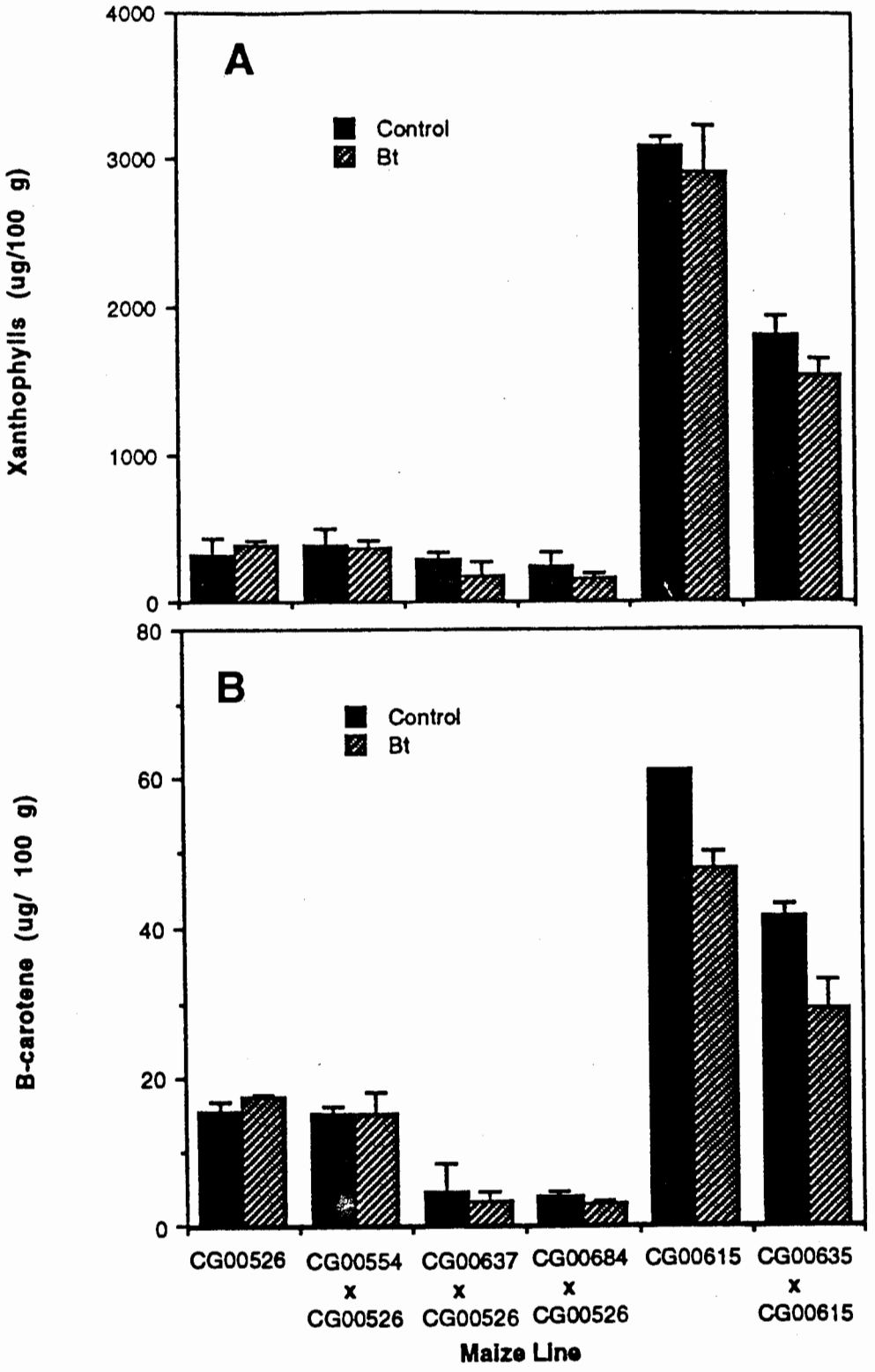

Figure 7. Graphic representation of mean carotenoid values given in Table 2 for kernels of two *Bt* maize inbreds and four hybrids (hatched bars) and the corresponding nontransgenic inbreds or hybrids (solid bars). Standard deviations are indicated. Panel A. Xanthophyll levels; Panel B. β-carotene levels.

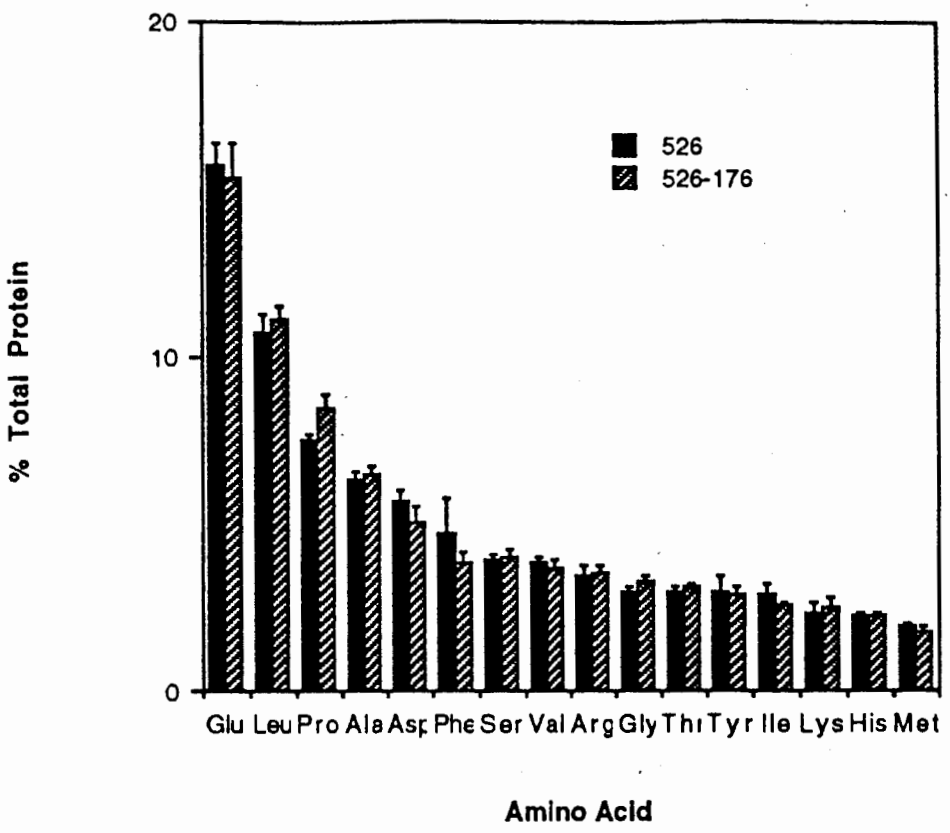

Figure 8. Graphic representation of mean amino acid values given in Table 3 for kernels of *Bt* maize inbred CG00526-176 (hatched bars)and the corresponding nontransgenic inbred CG00526 (solid bars). Standard deviations are indicated.

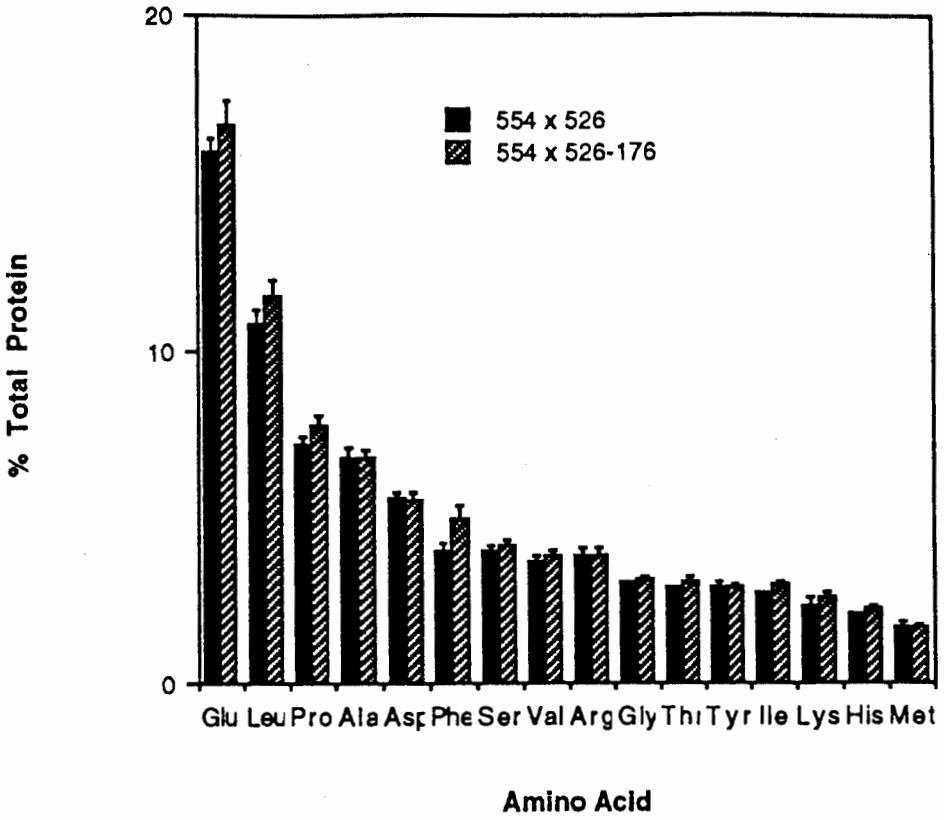

Figure 9. Graphic representation of mean amino acid values given in Table 3 for kernels of *Bt* maize hybrid CG00554 x CG00526-176 (hatched bars)and the corresponding nontransgenic hybrid CG00554 x CG00526 (solid bars). Standard deviations are indicated.

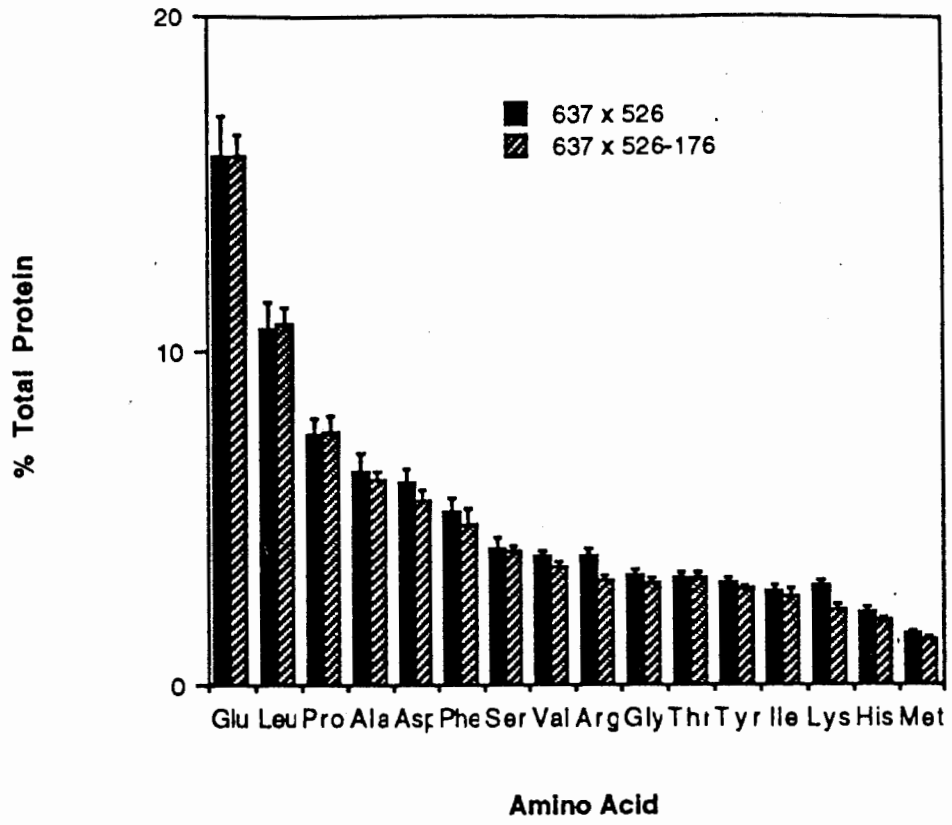

Figure 10. Graphic representation of mean amino acid values given in Table 3 for kernels of *Bt* maize hybrid CG00637 x CG00526-176 (hatched bars)and the corresponding nontransgenic hybrid CG00637 x CG00526 (solid bars). Standard deviations are indicated.

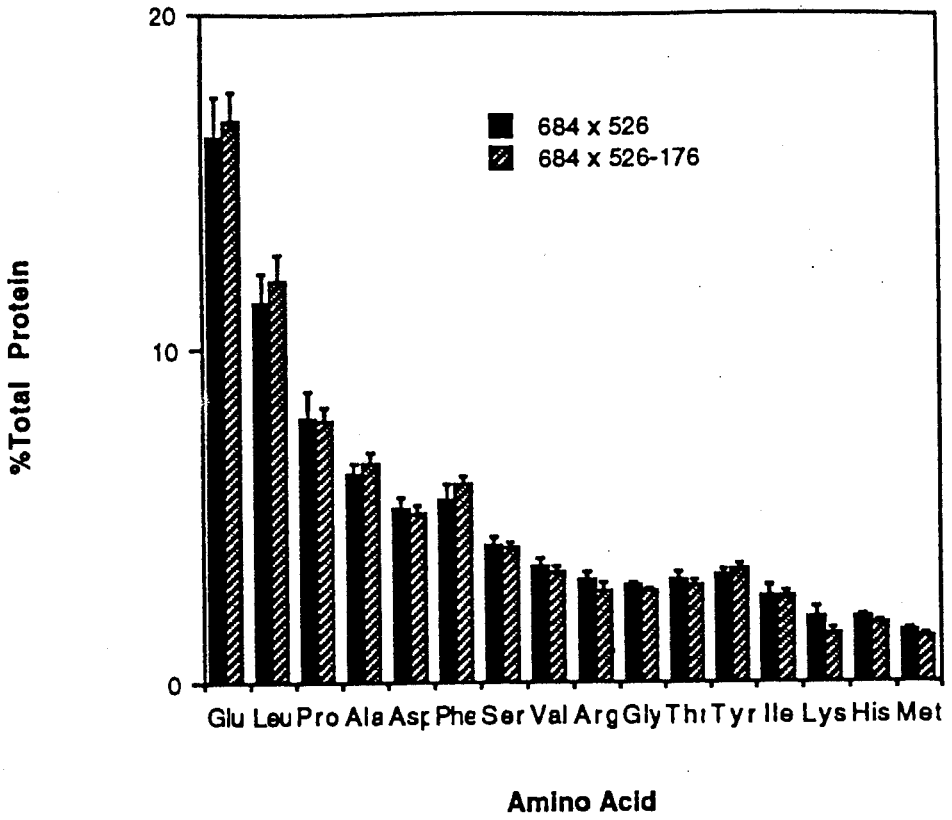

Figure 11. Graphic representation of mean amino acid values given in Table 3 for kernels of *Bt* maize hybrid CG00684 x CG00526-176 (hatched bars)and the corresponding nontransgenic hybrid CG00684 x CG00526 (solid bars). Standard deviations are indicated.

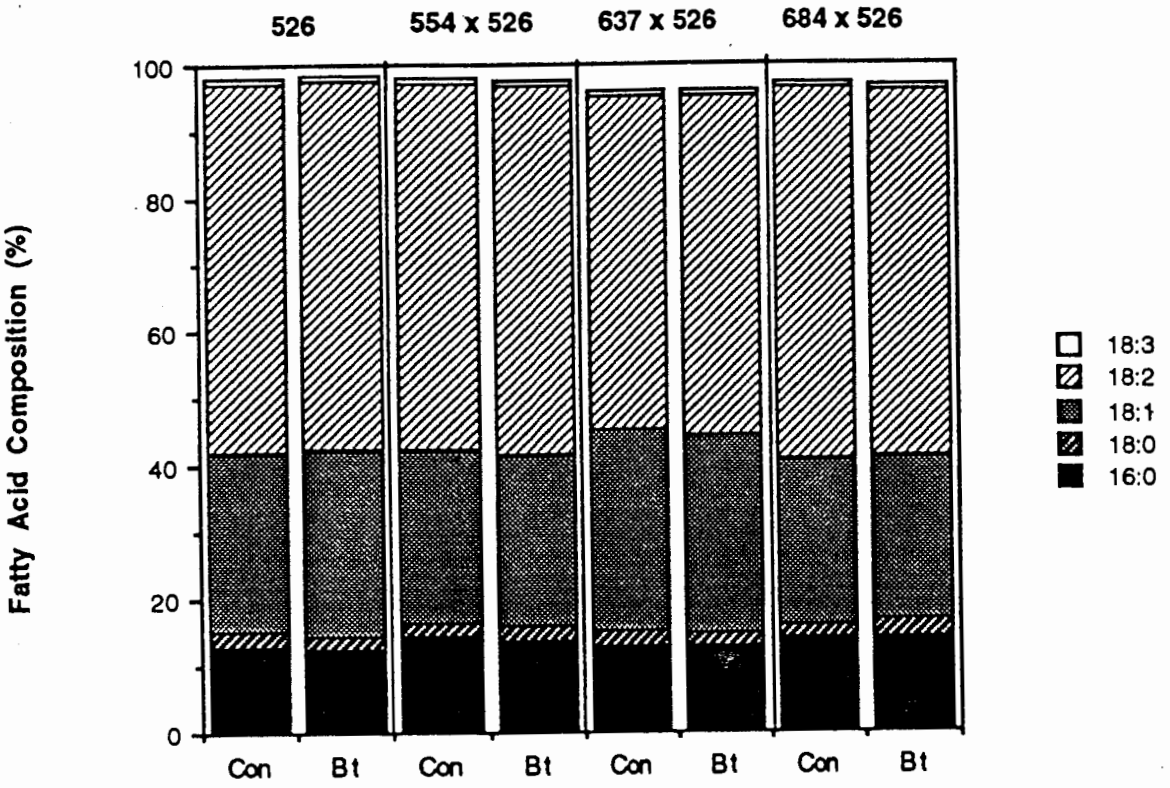

Figure 12. Graphic representation of mean fatty acid values given in Table 4 for kernels of one *Bt* maize inbred and four hybrids ("Bt") and the corresponding nontransgenic inbreds or hybrids ("Con"). Abbreviation for maize genotypes are as follows: 526 = CG00526; 554 x 526 = CG00554 x CG00526; 637 x 526 = CG00637 x CG00526; 684 x 526 = CG00684 x CG00526.

CHAPTER 10

AGRONOMIC PERFORMANCE OF *Bt* MAIZE

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Lists of Tables, Figures, and Appendices

10. AGRONOMIC PERFORMANCE OF *Bt* MAIZE

Summary

A total of nineteen genetically different maize lines derived from backcrosses of elite inbreds to CG00526-176 were field tested in the United States in 1992, 1993, and 1994. The performance of the transgenic germplasm was compared with isogenic or closely related non-transgenic germplasm. The trials were monitored by plant breeders and product development agronomists highly experienced in commercial maize production. The plants were evaluated for incidence of disease and pests, reactions to herbicides and pesticides, morphological characteristics, and yield. *Bt* maize plants exhibited significantly increased yields, and the general health of the plants appeared to benefit from the lack of European corn borer damage due to expression of CryIA(b) protein. Further, there was no indication that the transformation process or expression of the *cryIA(b)* or *bar* genes increased the weediness potential of maize plants derived from event 176.

A. Yield Performance

This report is a summary of major field tests in which Ciba Seeds has tested the efficacy of *Bt* maize (176 ICP) for control of ECB and has measured associated changes in hybrid yield and other agronomic traits. Four categories of tests will be reviewed:

1. **Initial efficacy evaluation.** This field test was conducted in 1992 and was described by Koziel *et al.* (Appendix 1).
2. **Gene efficacy studies.** Experiments were conducted in 1993 and 1994 to assess the effect of 176 ICP on yield and other agronomic traits. The 1993 experimental results were summarized by Christensen *et al.* (Appendix 2).
3. **Insecticide comparisons.** A 1994 Ciba trial compared a hybrid containing the *cryIA(b)* gene with several commonly used insecticides for control of ECB. In addition, Ciba included a 176 ICP hybrid in a 1994 insecticide evaluation trial conducted by the University of Illinois.
4. **Performance evaluation strip tests.** These 1994 tests compared the performance of isogenic hybrids with and without the 176 ICP gene in naturally infested field conditions.

Initial Efficacy Evaluation

- **Overview**

The purpose of this field test was to evaluate whether plants containing the *cryIA(b)* gene suffered less damage when challenged with extremely high artificial ECB infestations. A full description of the study has been published by Koziel, *et al.* (*Bio/Technology* 11: 194-200; (see Appendix 1). The major conclusion of the study was that a very high level of protection from ECB damage was maintained in 176 ICP plants during repeated heavy infestations of this pest.

- **Procedures**

Progeny of crosses of the original transgenic inbred with various other elite lines were infested with 300 larvae per week for a total of eight weeks. The first four infestations corresponded with the first generation ECB infestation period, while the latter four

infestations corresponded with the second generation period. These levels of infestation were approximately 12 to 96 times the economic threshold for second generation ECB infestation. The leaf feeding damage typical of first generation ECB was visually evaluated, while second generation ECB damage was assessed by measuring stalk tunnel length.

- **Results and Discussion**

Almost all 176 ICP plants showed no more than slight "window pane" first generation ECB damage to the epidermal layer of leaf tissue. Control plants typically had elongated lesions and broken midribs. As the season progressed, control plants senesced and disintegrated while 176 ICP plants remained green. At the end of the season, most 176 ICP plants had 0 to 5 cm tunneling damage, while mean tunnel length in control plants ranged from 28 to 113 cm. These results provided the first field demonstration of the high level of ECB control provided by the 176 ICP.

Gene Efficacy Studies

- **Overview**

In 1993 and 1994, multilocation studies were organized to further measure the degree of protection provided by 176 ICP plants against ECB damage. A particular goal was to assess the effect of this protection on yield and other agronomic traits important to farmers. The procedures and results of the 1993 studies were described by Christensen *et al.* (Proceedings of the Forty-Eighth Annual Corn and Sorghum Research Conference, pp. 43-52. American Seed Trade Association. Washington, D.C. 1993; See Appendix 2). This summary provides additional data from the 1993 study plus results from 1994 studies. The conclusions of these studies are 1) ECB can cause significant yield loss and negatively affect other important agronomic traits, 2) ECB damage is highly variable, resulting from the interactions among ECB, environments and hybrids, 3) hybrids containing the 176 ICP demonstrate high levels of protection against ECB damage and consequent yield loss and 4) in the absence of ECB pressure 176 ICP hybrids yield the same as isogenic hybrids without 176 ICP.

- **Procedures**

Inbred CG00526 plants from event 176 were self-pollinated and progeny tested to identify homozygous isolines which either contained the Bt gene (Bt+) or did not contain the Bt gene (Bt-). These isoline selections were then crossed to elite tester inbreds to produce hybrid seed for field tests. In addition, standard non-transformed plants of inbred CG00526, designated wild type (WT), were crossed to the same testers to produce a hybrid which did not contain the Bt gene and was not derived from transformed plants.

In 1993, the three versions of one hybrid (Bt+, Bt- and WT) were tested for performance under ECB pressure, utilizing a split-block type experiment with infestation treatments as main plots and hybrids as sub-plots. In 1994, the Bt+ and WT versions of two hybrids were tested with the same experimental design. The experiment contained three replicates of each treatment. Four-row plots were planted in 1993, and 6-row plots were used in 1994. Only the center two rows of each plot were used for treatments and data collection in order to reduce possible confounding effects due to migration of ECB larvae. In 1993, all four rows of the experimental plots were detasseled in order to include the trials with other corn plantings and still satisfy regulatory requirements for isolation of transgenic plants. Adequate pollen for ear formation in the experimental plots was provided by planting non-

detasseled borders of normal hybrids on each side of every experimental plot. In 1994, plots were not detasseled, but were instead spatially isolated from other corn fields by at least 660 feet.

Five whole-plot treatments were used:

1) ECB1: Two applications of 100-150 ECB larvae at the mid-whorl stage of growth to simulate heavy first generation ECB infestation, followed by insecticide protection as needed for second generation infestation;

2) ECB1+2: Two applications of 100-150 ECB larvae at the mid-whorl stage of growth followed by two applications of 100-150 larvae at mid-pollen shed to simulate heavy first and second generation infestation;

3) ECB2: Insecticide treatment as needed for natural first generation ECB, followed by two applications of 100-150 ECB larvae at mid-pollen shed to simulate heavy second generation infestation;

4) NATURAL: No applications of ECB or insecticide in order to measure the effects of natural ECB infestations;

5) PROTECTED: Insecticide applied as needed throughout the season in order to provide an uninfested control for all genotypes. A commercial synthetic pyrethroid insecticide was used for all insect protection applications.

Data was collected from five locations in the 1993 season (Goehner, NE; Marion, IA; Shelbyville, IL; and Bloomington, IL (Center Farm and North Point) and eight locations in the 1994 season (Bluffton, IN; Farmer City, IL; Leroy, IL; Bloomington, IL; Shelbyville, IL; Goehner, NE; Marion, IA; and Owatonna MN) (USDA permits #93-014-01r, #94-056-06N, and 94-076-10N). Data were collected for yield, grain moisture at harvest (MOIST), test weight (TSTWT), % plants not broken below the ear (%ERCT), % plants not broken after a push (%PUSH), plant intactness above the ear (INTCT), % dropped ears (%DEAR), % late season staygreen (%GRN), late appearance (APRLR), final population (FPOPN), first generation ECB damage (ECB1), second generation ECB damage (ECB2), inches of ECB tunneling (INCHTNL), # ECB entry holes in the stalk (#HOLS), stalk rot rating (STKROT) and % moldy ears (PERERO). All visually rated traits (INTCT, APRLR, ECB1, ECB2, STKROT), were evaluated with scores of 1 - 9, with 1 indicating a perfect appearance.

Analyses of variance were obtained for all traits. All effects except replicates and locations were considered fixed. The significance level p=0.05 was used for all data comparisons.

• Results and Discussion

Data from 1993 and 1994 is presented in Table 1A and Table 1B for hybrids 5506BTX (Bt+) and 5506X (WT). Data was also collected in 1994 for a second set of hybrids, 4806BTX (Bt+) and 4806X (WT) (Table 2A and 2B). In each table, hybrid means within each treatment are shown averaged over the entire set of locations and for each individual location included in the study. Data was not collected for all traits at all locations.

The Bt+ hybrids had only very limited feeding damage due to ECB1 infestations, whereas the WT hybrids showed extensive damage. These differences, however, were not reflected in the yield results. There were no differences in yield performance of the two genotypes

within the ECB1 treatment, and there was no difference between treatment means for ECB1 and PROTECTED. For this set of locations there was no adverse yield effect of first generation infestations. The exact reasons for the results are unclear. Previous studies have shown sizable yield losses due to first generation ECB (Bode and Calvin, 1990; Lynch, 1980).

The 5506BTX hybrid had significantly less visible ECB2 damage in the ECB1+2 and ECB2 treatments compared to its isogenic control (5506X). Measures of tunneling damage and entrance/exit holes also showed a consistent advantage for 5506BTX. Both the *Bt* and non-*Bt* 4806 hybrids had very little second generation damage compared to the 5506 hybrids. There is no obvious explanation for this finding, as the parents of 4806X are not naturally resistant to ECB. This hybrid was not well-adapted to the locations from which tunneling data was collected, and it senesced earlier than other hybrids in the trial, but it is not clear how this could have resulted in less tunneling damage.

Over two years the 5506BTX (Bt+) hybrid demonstrated a significant yield advantage over the 5506X (Bt-) in both the ECB1+2 and ECB2 treatments. Mean yield increase of 5506BTX in the ECB1+2 and ECB2 treatments was just over 15 bushels per acre, or about 15%, greater than the susceptible genotypes. However, results differed within single years. In 1993, the 5506BTX hybrid consistently yielded more than 5506X in both the ECB12 and ECB2 treatments, but in 1994 the two hybrids differed only in the ECB1+2 treatment. Similarly, in 1994 the 4806BTX (Bt+) hybrid yielded 11 bushels per acre more than 4806X in the ECB12 treatment (significant at the p=0.10 level), but there was no yield difference between 4806BTX and 4806X in the ECB2 treatment. Apparently, only the combination of ECB1 and ECB2 damage caused yield loss in 1994. ECB damage is affected by the interaction among ECB, the environment, and individual hybrids. This interaction can result in widely different patterns of damage across years. The results of these studies indicate that hybrids which contain 176 ICP provide protection against ECB damage regardless of when infestation occurs.

There were consistent trends, but not always statistically significant differences for other agronomic traits between the genotypes in all but the PROTECTED treatment. Hybrids which contained 176 ICP had better stalk strength, as demonstrated in % erect, % push and intactness scores. They also had higher test weight and better late-season staygreen.

The hybrids in the PROTECTED treatment were closely monitored throughout both the 1993 and 1994 growing seasons in order to discern any morphological or developmental differences. None were observed. All hybrids flowered during the same period and were equal for height and other morphological characteristics. There were no significant differences for yield among hybrids in the protected treatment. The evidence of this study is that, except for the advantages of ECB control, there was no negative effect of the inserted genes on the overall performance of hybrids which contained 176 ICP.

Insecticide Comparisons

- **Overview**

Data was collected from trials conducted by Ciba Seeds and by the University of Illinois. The purpose of the trials was to compare corn which contained the 176 ICP with commonly used insecticides for control of generation ECB. Two trials comparing first generation ECB control measures demonstrated that corn which contained 176 ICP was equal to or better than the tested insecticides. One trial comparing second generation

control measures was inconclusive due to poor survival of ECB larvae on untreated as well as treated plants.

Procedures

Ciba First Generation ECB Comparison A split-plot trial with the ECB infestation regime (Natural and Artificial infestation for ECB1) as main-plots and the ECB1 insecticide treatment (no insecticide treatment, granular applications of DIPEL® (*B.t. kurstaki*), POUNCE® (permethrin), or LORSBAN® (chlorpyrifos) on the isogenic control hybrid or the Bt hybrid) as the sub-plots was planted at 2 locations in Mclean County, IL. The main-plots were replicated 3 times and arranged as randomized complete blocks. Each experimental plot consisted of 4 rows of the appropriate hybrid with the middle two rows of each experimental plot being the experimental unit. Approximately one month after planting, all plants in plots comprising the artificial infestation regime were infested with neonate ECB larvae mixed with corn cob grits. On two consecutive days, approximately 100 larvae were placed into the whorl of each plant for a total of 200 larvae per plant. Two days after the last infestation, appropriate applications of granular formulations of the three compounds were made in both ECB infestation regimes. The *Bt* hybrid and the "no insecticide treatment" in both types of main-plots did not receive applications of these compounds. Approximately two weeks after infestation, all plots were rated visually for ECB1 damage. Also, ten plants in each plot were dissected to determine the percentage of plants which were infested with ECB. Analyses of variance were obtained using SAS. In the analysis of variance model, all effects except replicates and locations were considered fixed. All comparisons were made at the $p<0.05$ significance level.

Ciba First Generation ECB Comparison - Treatments

HYBRID	ECB INFESTATION	ECB1 PROTECTION
BT TRANSGENIC HYBRID	ECB1	Bt TRANSGENE
NORMAL HYBRID	ECB1	POUNCE 1.5G
NORMAL HYBRID	ECB1	LORSBAN 15G
NORMAL HYBRID	ECB1	DIPEL 10G
NORMAL HYBRID	ECB1	NONE
BT TRANSGENIC HYBRID	NATURAL	Bt TRANSGENE
NORMAL HYBRID	NATURAL	POUNCE 1.5G
NORMAL HYBRID	NATURAL	LORSBAN 15G
NORMAL HYBRID	NATURAL	DIPEL 10G
NORMAL HYBRID	NATURAL	NONE

University of Illinois First Generation ECB Comparison A randomized complete block design with four replications and 25 insecticide treatments was used at one location. Each plot was 10 x 65 feet (four rows of corn 65 feet long) spaced 30 inches apart. The two center rows of each plot were artificially infested and treated with insecticides. Two untreated check plots were included in each replication and were averaged for the ANOVA. A total of 30 plants (15 consecutive plants in each of the two middle rows) in each plot were artificially infested with two black head egg masses in the whorl of the plant. Corn plants were in the mid to late whorl stage at the time of infestation. Insecticides were applied five days after infestation. The Ciba *Bt* maize was not treated with insecticide. Evaluations were made three to four weeks post-treatment to

allow the ECB to complete their larval stage. The number of borer cavities, lengths of each cavity, and number of ECB larvae per plant were recorded for each of 20 plants that were artificially infested and statistical analyses were performed.

Ciba Second Generation ECB Comparison The general design of this experiment was the same as the Ciba First Generation Comparison, except that there were three ECB infestation regimes (Natural, Artificial Infestation for Second Brood Only, and Artificial Infestations for First and Second Broods). ECB infestations were made at the time of 50% pollen shed on leaves above and below the ear leaf. Data was collected for yield, harvest moisture (MOIST), test weight (TSTWT), number stalks broken below the ear (BROKN), number dropped ears (DREAR), % plants not broken after a push (PEPUSH), plant intactness above the ear (INTCT), final population (FPOPN), ECB2 damage (ECB2RT), stalk tunneling damage (TNWHPLT) and number of ECB entrance/exit holes (NUMHOLS).

Ciba Second Generation ECB Comparison

	ECB INFESTATION	ECB1 PROTECTION	ECB2 PROTECTION
BT TRANSGENIC HYBRID	ECB2	POUNCE 1.5G	NONE
NORMAL HYBRID	ECB2	POUNCE 1.5G	POUNCE FOLIAR
NORMAL HYBRID	ECB2	POUNCE 1.5G	LORSBAN FOLIAR
NORMAL HYBRID	ECB2	POUNCE 1.5G	DIPEL FOLIAR
NORMAL HYBRID	ECB2	POUNCE 1.5G	NONE
BT TRANSGENIC HYBRID	ECB1&2	NONE	NONE
NORMAL HYBRID	ECB1&2	POUNCE 1.5G	POUNCE FOLIAR
NORMAL HYBRID	ECB1&2	LORSBAN 15G	LORSBAN FOLIAR
NORMAL HYBRID	ECB1&2	DIPEL 10G	DIPEL FOLIAR
NORMAL HYBRID	ECB1&2	NONE	NONE
BT TRANSGENIC HYBRID	NATURAL	NONE	NONE
NORMAL HYBRID	NATURAL	POUNCE 1.5G	POUNCE FOLIAR
NORMAL HYBRID	NATURAL	LORSBAN 15G	LORSBAN FOLIAR
NORMAL HYBRID	NATURAL	DIPEL 10G	DIPEL FOLIAR
NORMAL HYBRID	NATURAL	NONE	NONE

- **Results and Discussion**

Data for combined and individual location treatment means from the Ciba first generation comparison is presented in Table 3 and Table 4. Preliminary, unpublished, data from the University of Illinois comparison is presented in Table 5, Table 6, and Table 7. In the Ciba study, all insecticide plots were significantly less infested than the untreated control. Although the level of control in the BTGENE treatment was not significantly different than other insecticide treatments, the BTGENE treatment was unique in infestation levels (0 %) and exhibited very low levels of ECB1 damage. In the University of Illinois study, the 176 ICP hybrid (*Bt* maize) was one of only two control treatments consistently in the lowest damage category for each trait. The *Bt* maize entry was significantly better than most other entries for each trait.

Data for combined and individual location treatment means from the Ciba Second Generation comparison is presented in Table 8 and Table 9. ECB insecticide treatments were different from the untreated control only for tunnels and number of holes in the ECB1+2 treatment. The 176 ICP hybrid had significantly fewer tunnels and holes than the untreated control in this treatment, but it was not different than other insecticide treatments. Overall, the lack of ECB damage in the untreated plots indicates that there was very low survival of second generation ECB in this experiment. Both locations of this experiment experienced very dry growing conditions during 1994, and may have contributed to poor survival of the neonate ECB larvae. Because of the low ECB infestation, this trial did not provide a good measure of the efficacy of the various control treatments.

The insecticide comparison experiments described in this section have similar, widely used, designs for evaluation of insecticides used for ECB control. The artificial ECB infestation is conducted at one point in time, and chemical applications are timed for maximum control of the infestation. This situation is unlikely in a grower's field, since egg masses are likely to be laid on corn plants over a period of time. Larvae hatch over a period of time, making the timing of chemical applications difficult. Therefore, the observed efficacy of commercial insecticides in these studies is unlikely to be routinely achieved in common practice. Hybrids containing 176 ICP are able to control ECB continuously during the primary ECB flight periods, and therefore offer an ECB control option which is not measured by these studies.

Performance Evaluation Strip Tests

- **Overview**

The purpose of this study was to compare the performance of isogenic hybrids with and without 176 ICP in numerous naturally infested large plots. Overall, hybrids with 176 ICP had a nearly 12 bushel advantage over normal counterparts. The 176 ICP hybrids also had relatively better performance for other important agronomic traits.

- **Procedures**

Two genotypes were planted in each plot, a hybrid which produced CryIA(b) protein and a normal isogenic version of the same hybrid. Single replications of each genotype were planted in large plots of four to eight rows spaced 2.5 - 3 feet apart. Each plot was 100 - 200 feet long. During the growing season data was collected for stand, visible corn borer damage and stay-green. Some portions of some plots were artificially infested with ECB. These portions of the plots were not used for harvest data collection. At harvest, data was collected from each plot for grain yield, test weight, moisture, ECB entry holes, dropped ears, % plants erect and % plants erect after a push. Harvest data was collected from five 1/1000 acre subsections of each large plot. Yield was determined within each subsection by hand harvesting ears from three predetermined plants (e.g., plants 7, 13, and 21 in the row). A paired-t test was conducted on the mean difference between the 176 ICP hybrids and the normal hybrids. All data available at the time of this report (42 plots) is summarized.

- **Results and Discussion**

Data for individual locations and mean hybrid differences is presented in Table 10. Across 42 locations, the *Bt* hybrids yielded an average 13.78 bushels/acre more than hybrids without 176 ICP. The 176 ICP hybrids also had lower moisture at harvest, higher test

weight, fewer dropped ears, and less stalk lodging than their non-*Bt* counterparts. All hybrid comparisons were highly significant ($p<0.01$) except for final population.

ECB infestation levels varied across the corn belt during 1994. While some of the performance evaluation strip tests received heavy natural infestations, other plots did not have an obvious infestation. Most university extension agents did not comment about ECB moth flights until late in the season. Thus, the ECB infestation level during 1994 was not exceptionally heavy. Despite this, the performance of 176 ICP corn in 1994 strip tests offers strong evidence of the benefits which these hybrids offer to growers, even under conditions of moderate ECB infestation.

- **Summary -Yield Studies**

The four categories of studies discussed in this report offer consistent evidence from the past three years of the field performance of corn with 176 ICP. Under both artificially and naturally infested conditions, hybrids with this protein are typically superior to hybrids without the 176 ICP. Under conditions in which ECB is not a factor for agronomic performance, the 176 ICP hybrids are equal to check hybrids. In comparisons with traditional insecticide controls for ECB, the 176 ICP hybrids are at least equal to chemical controls, and they offer the added benefit of convenient ECB control throughout the primary periods of potential infestation. ECB damage has traditionally been difficult to assess and control. Hybrids with 176 ICP offer both a standard for measurement of the cost of ECB damage and an improved option for reduction of that cost.

B. Morphological and Agronomic Characteristics

In 1994, the agronomic performance of three *Bt* hybrids and several breeding germplasm (*Bt*) lines was evaluated at 55 field sites in 18 states (Table 11). Sites throughout the corn growing regions of the continental United States and Hawaii were chosen to enable performance evaluations of the hybrids under diverse environmental conditions, as well as exposure to a broad group of pathogens and pests indigenous to the various geographic regions. At each site, the transgenic varieties and their genetically equivalent non-transgenic counterparts were monitored throughout the growing season by plant breeders and agronomists (mean experience: 14 years). In addition to regular inspections for disease and insect pests, qualitative comparisons for a large number of morphological and agronomic traits were made between the transgenic and non-transgenic germplasm. The parameters chosen for this comparison are those which are typically used by professional breeders and agronomists in the industry, and cover a broad range of characteristics that encompass the entire life cycle of the maize plant, including:

- stand establishment
- early plant vigor
- leaf orientation
- leaf color
- plant height
- root strength (lodging)
- silk date
- silk color
- ear height
- ear shape
- ear tipfill
- intactness
- dry ear weight
- tassel color
- tassel size
- yield
- reaction to insecticides
- reaction to fungicides

The observations of the agronomists and breeders have been assembled. Their overwhelming assessment is that except for tolerance to European corn borer and glufosinate ammonium herbicide (and the noted exceptions cited below), the performance of the *Bt* maize hybrids is indistinguishable from their non-transformed isogenic

counterparts. This was true for all three hybrids and breeding germplasm lines evaluated across all the geographic regions in this study.

In a separate study performed in 1994, seven *Bt* maize hybrids were planted in up to 10 locations in the continental United States, and compared to their non-transgenic counterpart for yield performance and a wide range of agronomic characteristics typically used by plant breeders in their evaluation of new commercial varieties. This study employed an insecticide spray program so the performance of the hybrids could be assessed in the absence of insect feeding pressure. The results from this study are summarized in Table 12. For the vast majority of the traits evaluated in this study, there were no statistically significant differences between the performance of the transgenic lines and their isogenic controls. While differences were discerned in some categories, there were no consistent trends in the data that would indicate that these differences were due to the presence of the transgenes. Rather, the variation is within the normal range that is experienced in these kinds of studies and is of no agronomic significance.

Except for the Hawaii and Florida locations, the field sites will lay fallow until spring of 1995, at which time the incidence of volunteers will be assessed. Observations from previous years' field trials indicate few, if any volunteer plants in these plots. Any that do arise are either plowed under, treated with glyphosate herbicide, or both (see Supplement I. Environmental Release Reports).

Following harvest, the Florida and Hawaii plots are irrigated on a regular basis to promote seed germination, and the fields are monitored for volunteers for a minimum of 60 days. During the 1992 and 1993 field trials, there were no volunteer maize plants present at the Hawaii station. During this same time period, a total of 13 volunteer plants were observed in our field plots in Florida. The Ciba Seeds agronomist responsible for this field site has indicated that this volunteer frequency is normal, regardless of the genetic background of the germplasm. In all the field trial evaluations of event 176 derived maize plants (inbreds and hybrids) in 1992, 1993, and 1994, where the experimental design allowed for volunteer assessments to be made, there has been no indication that the incidence of volunteer plants (weediness potential) has increased in event 176 derived plants as compared to non-transformed counterparts.

- **Disease and Pest Observations**

Incidence of fungal and bacterial disease common to maize occurred during these field trials in 1994. Disease pressure varied among the different pathogens, but no measurable difference between *Bt* maize hybrids and genetically comparable controls was seen (Table 13). The incidence of anthracnose and fusarium-incited stalk rot was greater in the non-*Bt* hybrids. Most of the lesions on these non-*Bt* plants emanated from areas of damage caused by ECB feeding. The open wounds resulting from insect feeding are an excellent environment for fungal spore germination. The initiation of disease at these wound sites is consistent with the known etiology of these diseases. The Hawaii and Florida field sites employed several fungicides (Tilt®, Dithane®, Maneb®) in some of their trials and there were no unusual responses of the transgenic lines to these materials.

As a component of the 1993 field study discussed earlier in this report, a comparative study evaluated the incidence of ear mold present on *Bt* hybrids and isogenic controls. At harvest, all ears from every plot were inspected for the presence or absence of ear mold. These inspections were performed by an agronomist with over 10 years experience in evaluating corn hybrids. The results of this study are presented in Figure 1. There was no

significant difference between the two hybrids (Bt+, Bt-) in the frequency of moldy ears regardless of the treatment performed on the plants. The presence of the transgenes provided no advantage nor disadvantage in terms of susceptibility to ear-rotting fungi.

Fungal infestation of ears from transgenic corn was further examined in a separate study. As an alternative to visual observations, the levels of aflatoxins present in stored kernels was measured. Differences in aflatoxin levels between transgenic and control lines would reflect an altered susceptibility to ear mold(s). Kernels were harvested from plants grown at the Molokai, HI field station during the fall and early winter of 1993, representing the homozygous *Bt* maize line CG00526-176, three hybrid crosses (CG00554 x CG00526-176, CG00684 x CG00526-176; CG00637 x CG00526-176) representing late, medium, and early maturity groups, respectively, and their isogenic counterparts. The seed from each corn line was stored under standard seed storage conditions ($10 \pm 1°$ C and $50 \pm 10\%$ relative humidity) for nine months. For each maize variety, five 100- g aliquots of seed were individually ground to a fine powder and shipped on dry ice to Southern Testing and Research Laboratories, Wilson, NC for aflatoxin analysis, using AOAC Method 991.31. The results from this study are presented in Table 14. For the maize inbred CG00526 and its homozygous *Bt* counterpart, one sample of five from each had detectable levels of aflatoxin B_1 and B_2. Hybrid CG00554 x CG00526 had one sample with detectable levels of B_1 while there was no detectable aflatoxin from all five samples from the transgenic line. Two samples from each of the CG00684 x CG00526 hybrids had measurable levels of aflatoxin. Both the transgenic and non-transgenic hybrids CG00637 x CG00526 had aflatoxin present in four of five samples. The inbred CG00637 has a history of inferior seed quality, reflected in a low germination frequency and high incidence of fungal infestation of the ear. The specific seed derived from the hybrid crosses used in the aflatoxin study were observed by a Ciba Seeds agronomist and confirmed to be of the inferior quality indicative of hybrids having CG00637 parentage. However, there was no apparent difference in the levels of aflatoxin present in the control hybrid seed and transformed hybrid seed. This was also true for the homozygous inbreds (CG00526; CG00526-176) as well as the two other hybrid pairs used in this study.

The data from these studies indicate that plants derived from event 176 are indistinguishable from controls with respect to susceptibility to fungal ear mold and aflatoxin levels in stored seed.

There was no discernible difference in non-ECB insect damage incurred by the transgenic and nontransgenic maize lines (Table 15). In some locations (Hawaii and Florida) a weekly insecticide spray program precluded any measurements regarding insect damage and population monitoring. A variety of insecticides was used (Asana®, diazinon, Lannate®, Metasystox®, Penncap®) at these locations and there were no unusual responses of the transgenic lines to these materials.

- **Summary -Morphological and Agronomic Characteristics**

The performance of *BT* maize inbreds and hybrids was evaluated in field trials throughout the United States in 1992, 1993, and 1994. The plants were scrutinized for a multitude of traits under diverse environmental conditions and agricultural practices. These studies indicate that expression of the *cryIA(b)* and *bar* genes in maize plants derived from event 176 results in no adverse effect on the growth, weediness potential, or other agronomic behavior of these plants.

C. References

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ABBREVIATIONS USED IN TABLES 1 - 10

- MOIST: grain moisture at harvest
- TSTWT: test weight
- %ERCT: % of plants not broken below the ear
- %PUSH: % of plants not broken after a push
- PEPSH: % of plants not broken after a push
- INTCT: plant intactness above the ear
- DREAR: % dropped ears
- %DEAR: % dropped ears
- %GRN: % of late season green
- APRLR: late appearance
- FPOPN: final population
- ECB1: first generartion ECB damage
- ECB2RT: second generation ECB damage
- ECB2: second generation ECB damage
- TNWHPLT: inches of ECB tunneling
- INCHTNL: inches of ECB tunneling
- #HOLS: # of ECB entry holes in stalk
- NUMHOLES: # of ECB entry holes in stalk
- STKROT: stock rot rating
- PEREROT: % ear rot
- INFREGM: Infestation regime
- VERSION: Genotype of Hybrid (*Bt* or non-*Bt*)

TABLE 1A. PERFORMANCE OF *BT* AND CONTROL HYBRID 5506 IN PLOTS ARTIFICIALLY INFESTED WITH ECB

TREATMENT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PEREROT
CONTROL	5506BTX	120.68	21.77	56.09	98.12	88.57	3.9	1.36	46.67	3.7	20522	1.3	1.56	1.50	0.33	5.60	21.33
	5506X	117.87	22.24	55.23	96.11	79.52	5.6	1.30	38.41	4.3	20806	2.2	3.89	4.72	0.62	5.80	23.67
ECB1	5506BTX	125.61	21.98	56.02	99.35	90.95	3.3	0.19	51.74	3.6	20534	1.1	2.11	1.94	0.38	5.13	19.33
	5506X	123.79	23.08	55.29	98.12	82.38	4.4	0.41	50.41	3.9	20692	5.2	4.11	4.02	1.00	5.20	17.60
ECB12	5506BTX	121.33	21.23	56.36	96.88	85.03	4.3	0.92	49.93	4.4	20543	1.1	3.67	2.80	0.89	5.80	44.27
	5506X	103.53	20.53	55.56	94.00	70.75	6.9	1.64	31.74	5.3	20764	4.9	7.11	15.45	2.78	5.73	55.20
ECB2	5506BTX	120.57	21.32	56.30	97.54	86.19	3.8	0.89	46.37	3.7	20801		3.56	4.33	1.04	6.00	41.53
	5506X	107.46	20.05	55.05	96.64	77.14	6.5	0.97	28.04	4.1	20933		6.44	13.91	2.44	5.80	51.00
PRTCT	5506BTX	126.59	21.41	55.98	98.55	90.95	3.4	0.33	49.19	3.4	20183	1.0	3.44	1.12	0.36	5.40	25.67
	5506X	126.38	22.63	55.02	97.92	88.57	4.6	0.37	49.33	3.9	21005	1.3	4.56	0.99	0.78	5.70	16.47
# Locations		12	12	8	11	7	12	11	9	3	12	7	3	8	3	5	5
LSD(0.05) - A		5.72	1.96	0.82	2.76	18.31	1.04	0.92	15.48	2.88	718	1.39	0.57	18.06	1.52	1.04	15.21
LSD(0.05) - B		8.20	1.60	0.89	2.40	14.54	0.98	1.07	12.54	1.64	563	1.04	1.72	14.49	0.88	1.12	14.78

A = FOR COMPARING VERSION MEANS IN THE SAME TREATMENT.
B = FOR COMPARING VERSION MEANS IN DIFFERENT TREATMENTS.

*BTX indicates Bt gene present, X is isogenic wild type control.

TABLE 1B. PERFORMANCE AT INDIVIDUAL LOCATIONS OF *BT* AND CONTROL HYBRID 5506 IN PLOTS ARTIFICIALLY INFESTED WITH ECB

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PERERC
BLUFFTON	CONTROL	5506BTX	128.11	25.70	53.74	100.00	100.00	3.0	0.00		2.0	21550	1.0					
		5506X	127.07	25.35	54.17	100.00	96.67	3.7	0.00		2.7	21550	1.0					
	ECB1	5506BTX	125.44	25.21	53.60	100.00	86.67	3.0	0.00		2.3	22063	1.0					
		5506X	125.29	25.82	51.90	100.00	80.00	3.7	0.00		3.0	20903	4.0					
	ECB12	5506BTX	101.29	25.30	51.19	100.00	96.67	4.0	0.00		3.3	22193	1.0					
		5506X	99.59	26.97	51.48	100.00	90.00	4.7	0.00		2.7	21933	4.7					
	ECB2	5506BTX	94.55	25.48	51.90	100.00	96.67	2.3	0.00		3.0	22323						
		5506X	109.63	26.18	50.98	100.00	100.00	3.3	0.00		2.7	22323						
	PRTCT	5506BTX	141.79	24.89	52.82	100.00	100.00	2.0	0.00		2.0	21677	1.0					
		5506X	124.97	25.76	53.46	100.00	100.00	2.3	0.00		2.3	21677	1.0					
		N	3	3	3	3	3	3	3	0	3	3	3	0	0	0	0	0
CFARM93	CONTROL	5506BTX	140.14	20.87		94.64		5.7	0.65	50.00		21532			4.13		6.33	36.67
		5506X	134.57	19.93		93.53		7.3	0.00	23.33		21957			11.87		6.67	46.67
	ECB1	5506BTX	135.82	21.60		99.31		3.7	0.72	60.00		20257			1.87		6.00	60.00
		5506X	132.69	21.33		98.64		4.3	0.64	56.67		20965			4.40		6.33	41.33
	ECB12	5506BTX	124.43	20.53		96.14		5.0	1.47	50.00		19124			3.47		5.33	73.33
		5506X	88.68	15.70		94.00		8.3	3.35	1.67		21390			14.00		7.00	76.00
	ECB2	5506BTX	124.35	21.63		93.71		6.0	4.66	33.33		20257			5.20		7.00	80.00
		5506X	89.76	15.03		96.13		8.3	1.96	1.67		21532			10.00		6.33	66.00
	PRTCT	5506BTX	139.77	21.40		97.94		4.0	0.00	50.00		20682			1.33		5.33	46.67
		5506X	141.01	21.50		96.59		5.0	0.65	56.67		21107			0.40		4.67	40.00
		N	3	3	0	3	0	3	3	3	0	3	0	0	3	0	3	3
FMRCITY	CONTROL	5506BTX	88.81	21.17	54.10	98.89	90.00	5.0	1.11	63.33		22240	1.0	2.00	0.13	0.33		
		5506X	86.54	23.17	53.53	97.77	86.67	6.3	5.00	63.33		22240	1.0	4.33	0.37	0.73		
	ECB1	5506BTX	108.91	19.85	54.52	100.00	100.00	3.3	0.00	66.67		22240	1.0	1.33	0.23	0.33		
		5506X	110.77	19.89	55.45	98.33	86.67	5.3	1.11	63.33		22240	6.7	3.67	0.80	1.00		
	ECB12	5506BTX	138.52	19.21	55.87	98.89	86.67	4.3	1.11	70.00		22240	1.0	3.67	0.73	1.33		
		5506X	112.76	19.80	54.95	98.89	90.00	5.0	1.11	70.00		22240	4.0	5.67	1.70	2.73		

continued

Table 1B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PERERC
FMRCITY	ECB2	5506BTX	122.46	18.85	55.66	98.89	76.67	3.0	0.00	70.00		22240		3.33	1.27	1.47		
		5506X	111.35	19.58	55.02	97.22	60.00	6.3	0.00	66.67		22240		6.33	3.40	2.87		
	PRTCT	5506BTX	140.70	20.36	54.31	100.00	90.00	3.0	0.55	70.00		22240	1.0	1.67	0.27	0.53		
		5506X	115.50	21.90	54.10	99.44	86.67	5.7	0.55	70.00		22240	1.0	3.67	0.20	0.93		
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3	0	0
FUNKFARM	CONTROL	5506BTX	150.85	18.55	53.89	100.00	93.33	4.7	0.00	70.00		22240	1.0	1.00	0.30	0.53		
		5506X	146.66	19.34	52.68	100.00	93.33	5.7	0.55	70.00		22240	3.3	4.00	0.40	0.33		
	ECB1	5506BTX	145.75	18.56	53.74	100.00	93.33	4.7	0.00	80.00		22240	1.0	4.00	0.50	0.80		
		5506X	143.09	19.11	52.96	98.89	80.00	6.3	0.00	66.67		22240	5.7	6.67	0.50	1.33		
	ECB12	5506BTX	140.60	18.30	54.10	100.00	100.00	5.7	0.55	80.00		22240	1.0	4.67	0.27	0.87		
		5506X	147.64	19.14	52.32	99.44	63.33	7.0	0.00	63.33		22240	6.0	8.67	1.37	2.53		
	ECB2	5506BTX	146.64	18.38	53.88	98.33	83.33	5.3	0.00	73.33		22240		5.33	0.47	0.87		
		5506X	141.10	19.28	52.47	99.44	83.33	6.0	0.00	63.33		21993		6.67	1.43	1.60		
	PRTCT	5506BTX	151.11	18.30	54.02	98.33	93.33	5.7	0.00	76.67		22240	1.0	5.33	0.17	0.27		
		5506X	133.66	18.67	53.18	100.00	80.00	8.7	0.55	73.33		21993	2.0	6.67	0.43	1.00		
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3	0	0
GOEHN93	CONTROL	5506BTX	69.26	25.53		98.85		2.3	0.00	0.00		12342			1.33		5.00	16.67
		5506X	66.74	25.53		88.11		5.3	1.08	0.00		13068			9.73		5.00	3.33
	ECB1	5506BTX	63.98	24.70		97.62		2.3	0.00	0.00		11616			10.27		5.00	3.33
		5506X	72.56	26.03		93.99		3.7	0.00	1.67		12923			19.60		4.67	0.00
	ECB12	5506BTX	63.59	22.87		90.93		3.7	2.02	0.00		13068			13.20		5.67	26.67
		5506X	40.36	18.43		92.43		7.7	2.15	0.00		13213			54.53		6.33	26.67
	ECB2	5506BTX	73.10	24.77		100.00		3.3	0.00	0.00		13358			21.07		4.67	27.67
		5506X	41.20	17.43		92.98		8.7	3.03	0.00		14375			51.07		6.00	44.00
	PRTCT	5506BTX	76.75	25.70		100.00		2.0	1.04	1.67		13068			2.80		5.33	0.00
		5506X	89.22	24.83		94.69		3.0	0.00	1.67		13794			4.40		5.33	0.00
		N	3	3	0	3	0	3	3	3	0	3	0	0	3	0	3	3

continued

Table 1B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PERI
GOEHNER	CONTROL	5506BTX	165.30	16.45	60.34	99.20	90.00	3.0	0.00		3.3	21177	1.0					
		5506X	154.55	17.51	58.78	97.61	43.33	5.3	0.00		4.3	21520	1.7					
	ECB1	5506BTX	161.00	16.97	59.34	100.00	100.00	2.7	0.00		2.7	21520	1.0					
		5506X	148.62	18.50	57.50	95.23	83.33	4.3	0.00		3.0	21520	4.7					
	ECB12	5506BTX	162.82	16.81	59.98	96.78	93.33	3.7	0.00		3.7	21177	1.0					
		5506X	132.89	16.36	60.33	90.47	40.00	7.0	0.79		6.7	21520	3.3					
	ECB2	5506BTX	152.55	16.32	60.05	100.00	96.67	2.7	0.79		3.0	21520						
		5506X	172.26	17.75	58.00	100.00	76.67	4.0	0.00		3.7	21520						
	PRTCT	5506BTX	165.36	17.31	58.49	99.20	96.67	2.3	0.00		2.3	21350	1.0					
		5506X	168.94	18.72	56.79	100.00	100.00	2.7	0.00		3.0	21520	1.0					
		N	3	3	3	3	3	3	3	0	3	3	3	0	0	0	0	(
LEROY	CONTROL	5506BTX	104.40	23.17	53.25	100.00	100.00	3.0	0.00	73.33		22240	1.0	1.67	0.17	0.13		
		5506X	109.28	25.04	51.26	100.00	96.67	4.7	0.00	70.00		22240	3.3	3.33	0.50	0.80		
	ECB1	5506BTX	123.42	22.37	52.89	100.00	96.67	3.3	0.00	73.33		22240	1.0	1.00	0.00	0.00		
		5506X	120.81	23.79	53.11	99.44	96.67	4.7	0.55	76.67		22240	6.3	2.00	0.50	0.67		
	ECB12	5506BTX	116.38	21.28	53.60	100.00	83.33	3.3	0.55	70.00		22240	1.0	2.67	0.17	0.47		
		5506X	104.33	20.70	54.03	98.33	73.33	6.7	0.00	56.67		22240	6.0	7.00	1.33	3.07		
	ECB2	5506BTX	123.74	20.03	54.59	100.00	100.00	2.3	0.00	66.67		22240		2.00	0.53	0.80		
		5506X	96.48	20.42	53.67	100.00	83.33	8.0	0.00	56.67		22240		6.33	1.73	2.87		
	PRTCT	5506BTX	105.13	21.72	53.67	98.33	96.67	5.3	0.55	70.00		22240	1.0	3.33	0.13	0.27		
		5506X	103.93	23.63	52.32	100.00	93.33	5.0	0.00	70.00		22240	1.0	3.33	0.20	0.40		
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3	0	(
MARION	CONTROL	5506BTX	154.32	18.29	59.20	98.10	46.67	7.3	0.54	21.67	5.7	26937	1.0					
		5506X	153.63	17.65	59.98	98.78	40.00	8.3	0.00	20.67	6.0	27423	3.7					
	ECB1	5506BTX	152.48	19.46	60.97	98.87	60.00	7.0	0.00	30.67	5.7	27260	1.0					
		5506X	156.97	19.97	59.27	97.55	50.00	7.7	0.00	22.00	5.7	27423	5.3					
	ECB12	5506BTX	148.11	17.90	61.61	95.54	36.67	8.3	0.57	32.67	6.3	28713	1.0					
		5506X	129.59	19.83	58.99	94.04	40.00	8.0	0.62	25.67	6.7	27100	4.3					
	ECB2	5506BTX	153.45	17.61	60.76	98.32	50.00	8.0	0.00	35.67	5.0	29037						
		5506X	150.40	17.41	59.06	96.33	36.67	8.7	0.00	24.00	6.0	26613						

continued

Table 1B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PERERC
MARION	PRTCT	5506BTX	156.23	18.17	59.84	95.82	60.00	6.3	0.00	29.33	6.0	26773	1.0					
		5506X	161.81	19.21	59.13	97.22	60.00	8.7	0.00	24.00	6.3	28710	1.7					
		N	3	3	3	3	3	3	3	3	3	3	3	0	0	0	0	0
MARION93	CONTROL	5506BTX	70.79	24.17		100.00		3.3	0.00	61.67		19037			1.18		6.00	6.67
		5506X	58.59	22.67		95.83		6.7	0.98	31.67		19360			5.69		7.67	10.00
	ECB1	5506BTX	82.11	25.07		100.00		3.7	0.00	68.33		19037			0.00		3.67	6.67
		5506X	83.67	27.73		100.00		5.3	0.83	73.33		19360			1.09		4.00	6.67
	ECB12	5506BTX	75.94	25.33		99.12		4.3	0.98	66.67		18553			0.56		6.00	10.00
		5506X	56.71	23.07		95.81		7.0	4.96	18.33		19360			13.82		6.33	33.33
	ECB2	5506BTX	74.97	23.60		97.30		5.0	0.81	51.67		18876			0.60		6.00	16.67
		5506X	46.05	20.00		95.75		7.0	0.00	6.67		19037			8.76		6.67	40.00
	PRTCT	5506BTX	61.60	23.20		100.00		3.7	0.00	56.67		17585			0.00		5.33	16.67
		5506X	76.92	24.73		99.10		5.0	0.00	56.67		19037			0.00		6.00	17.33
		N	3	3	0	3	0	3	3	3	0	3	0	0	3	0	3	3
NPOINT93	CONTROL	5506BTX	104.69	19.50	58.17	94.29		3.7	2.14	73.33		19222			3.73		6.00	23.33
		5506X	105.32	18.93	57.00	91.95		7.0	2.90	60.00		18945			7.60		5.00	36.67
	ECB1	5506BTX	125.23	19.67	56.83	98.52		2.7	0.67	80.00		19222			1.73		6.33	26.67
		5506X	126.20	21.00	56.00	98.57		2.7	0.68	83.33		19637			3.47		6.00	33.33
	ECB12	5506BTX	114.09	19.00	58.17	94.78		3.7	1.48	76.67		18669			2.93		6.67	63.33
		5506X	96.73	18.40	57.33	79.58		7.7	3.43	50.00		19637			23.33		4.33	83.33
	ECB2	5506BTX	122.99	19.07	57.33	96.39		3.0	0.00	80.00		18945			3.47		6.33	53.33
		5506X	95.32	18.57	56.83	95.07		8.0	1.45	33.33		19637			23.73		5.00	80.00
	PRTCT	5506BTX	112.79	19.77	58.00	99.15		1.3	0.00	86.67		17424			3.20		5.33	46.67
		5506X	125.43	22.53	55.67	94.33		2.3	0.00	86.67		19498			2.27		6.00	20.00
		N	3	3	3	3	0	3	3	3	0	3	0	0	3	0	3	3
SHELBVLE	CONTROL	5506BTX	163.25	26.32	56.01		100.00	2.3				19633	1.0					
		5506X	159.64	29.81	54.45		100.00	2.0				19633	1.0					
	ECB1	5506BTX	162.61	25.98	56.22		100.00	1.7				19633	1.3					
		5506X	148.59	27.88	56.15		100.00	3.0				19217	4.7					

continued

Table 1B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS	STKROT	PERERC
SHELBVLE	ECB12	5506BTX	150.78	25.60	56.37		98.52	2.0				19217	1.0					
		5506X	142.38	27.48	55.02		98.61	6.0				19217	3.3					
	ECB2	5506BTX	146.61	26.45	56.22		100.00	1.7				19217						
		5506X	138.00	27.37	54.38		100.00	2.0				19217						
	PRTCT	5506BTX	149.79	25.63	56.65		100.00	2.0				19080	1.0					
		5506X	158.37	28.54	55.51		100.00	2.0				19910	1.0					
		N	3	3	3	0	3	3	0	0	0	3	3	0	0	0	0	0
SHELBY93	CONTROL	5506BTX	108.23	21.47		95.40		4.0	10.50	6.67		18115			0.98		4.67	23.33
		5506X	111.87	21.93		93.64		5.0	3.81	6.67		19498			1.58		4.67	21.67
	ECB1	5506BTX	120.53	24.27		98.53		2.0	0.69	6.67		19083			0.92		4.67	0.00
		5506X	116.22	25.97		98.64		2.3	0.64	10.00		19637			1.81		5.00	6.67
	ECB12	5506BTX	119.42	22.60		93.48		3.3	1.36	3.33		19083			1.10		5.33	48.00
		5506X	90.70	20.43		90.97		8.3	1.63	0.00		19083			13.52		4.67	56.67
	ECB2	5506BTX	111.50	23.67		90.02		3.0	3.52	6.67		19360			2.02		6.00	30.00
		5506X	97.97	21.60		90.13		8.0	4.20	0.00		20466			11.18		5.00	25.00
	PRTCT	5506BTX	118.04	20.47		95.30		3.7	1.45	1.67		17839			1.02		5.67	18.33
		5506X	116.82	21.50		95.79		5.3	2.27	5.00		20328			0.00		6.50	5.00
		N	3	3	0	3	0	3	3	3	0	3	0	0	3	0	3	3
LSD(0.05) - A			19.83	6.78	2.33	9.15	48.45	3.6	3.04	46.44	5.0	2488	3.9	1.0	31.27	2.63	2.33	34.02
LSD(0.05) - B			28.40	5.53	2.53	7.97	38.46	3.4	3.55	37.62	2.8	1952	2.9	3.0	25.10	1.52	2.51	33.05

A = FOR COMPARING WITHIN LOCATION VERSION MEANS IN THE SAME TREATMENT.
B = FOR COMPARING WITHIN LOCATION VERSION MEANS IN DIFFERENT TREATMENTS.

TABLE 2A. PERFORMANCE OF *BT* AND CONTROL HYBRID 4806 IN PLOTS ARTIFICIALLY INFESTED WITH ECB

TREATMENT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS
CONTROL	4806BTX	123.39	19.73	56.35	86.84	87.41	5.6	0.33	49.47	3.4	22661	1.1	3.67	0.62	0.76
	4806X	124.34	21.10	55.23	86.30	84.07	6.5	0.69	45.60	4.4	22336	2.3	3.78	1.10	1.33
ECB1	4806BTX	129.01	19.44	56.89	86.86	90.00	4.6	0.55	50.93	3.6	23213	1.1	4.22	0.52	0.53
	4806X	123.27	20.82	55.73	86.04	85.77	5.9	0.32	47.73	3.9	22753	5.7	4.11	0.83	1.22
ECB12	4806BTX	120.18	19.11	56.78	86.24	82.59	6.0	1.02	48.47	4.7	22658	1.1	7.44	0.63	1.00
	4806X	108.65	19.74	56.02	85.79	71.08	7.9	0.58	38.73	5.4	22608	5.2	7.56	2.01	2.36
ECB2	4806BTX	124.46	19.43	56.33	86.29	90.96	5.4	0.45	50.93	3.6	22684		6.78	1.49	1.60
	4806X	122.39	20.47	55.83	85.46	84.28	6.3	0.60	45.47	4.4	22568		7.56	2.27	2.42
PRTCT	4806BTX	123.34	19.56	56.17	86.71	92.59	4.9	0.08	52.73	3.9	22899	1.0	5.89	0.30	0.47
	4806X	128.51	21.45	55.29	86.63	89.26	4.9	0.96	52.07	4.2	22773	1.1	6.00	0.69	1.04
	# Loc.	9	9	9	7	9	9	7	5	3	8	11	3	3	3
LSD(0.05) - A		13.62	0.98	0.87	2.57	15.51	1.47	1.54	8.75	2.96	593	0.99	4.21	1.45	2.10
LSD(0.05) - B		10.74	0.89	0.82	1.99	12.00	1.21	1.26	8.57	1.80	659	0.81	2.46	0.99	1.15

A = FOR COMPARING VERSION MEANS IN THE SAME TREATMENT.
B = FOR COMPARING VERSION MEANS IN DIFFERENT TREATMENTS.

TABLE 2B. PERFORMANCE AT INDIVIDUAL LOCATIONS OF *BT* AND CONTROL HYBRID 4806 IN PLOTS ARTIFICIALLY INFESTED WITH ECB

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS
BLUFFTON	CONTROL	4806BTX	116.24	22.87	52.68	100.00	100.00	4.0	0.00		2.7	21933	1.0			
		4806X	124.34	23.40	52.32	100.00	100.00	5.0	0.57		2.7	22193	1.0			
	ECB1	4806BTX	111.19	21.79	52.54	100.00	96.67	5.0	1.15		3.3	22450	1.0			
		4806X	113.57	23.29	52.54	99.41	96.67	6.0	0.00		3.3	21810	4.0			
	ECB12	4806BTX	107.66	22.47	52.61	100.00	100.00	5.7	0.00		3.7	22323	1.0			
		4806X	87.54	23.70	51.47	100.00	93.33	7.3	0.00		4.0	19097	4.7			
	ECB2	4806BTX	106.37	21.41	53.25	98.80	96.67	5.3	1.23		3.3	21030				
		4806X	107.00	22.68	51.55	99.41	86.67	5.3	0.00		4.0	21807				
	PRTCT	4806BTX	115.86	22.10	53.18	99.37	93.33	2.7	0.00		3.0	21807	1.0			
		4806X	112.66	23.14	52.04	99.38	100.00	3.3	0.62		2.7	20647	1.0			
		N	3	3	3	3	3	3	3	0	3	3	3	0	0	0
FMRCITY	CONTROL	4806BTX	73.73	18.00	56.08	96.67	80.00	7.0	0.00	56.67		22240	1.0	4.00	0.57	0.67
		4806X	81.79	19.09	54.52	98.20	80.00	8.0	0.00	50.00		20880	1.0	3.00	1.33	1.47
	ECB1	4806BTX	84.08	16.93	57.50	98.33	86.67	5.3	0.00	60.00		22240	1.0	3.33	0.67	0.40
		4806X	93.00	18.05	56.58	99.44	90.00	6.0	0.55	60.00		22240	6.7	3.00	1.30	1.40
	ECB12	4806BTX	71.11	16.77	57.00	97.78	66.67	7.7	5.55	60.00		22240	1.0	6.00	0.93	0.80
		4806X	97.61	17.53	56.36	97.77	90.00	8.7	0.00	60.00		22240	4.0	7.00	3.07	3.07
	ECB2	4806BTX	85.01	16.30	57.57	96.66	86.67	7.7	0.55	60.00		22240		7.00	2.43	1.73
		4806X	104.03	17.39	56.86	96.11	93.33	7.0	0.00	63.33		22240	.	6.67	3.57	3.40
	PRTCT	4806BTX	77.09	17.36	56.86	96.66	100.00	7.0	0.00	60.00		22240	1.0	7.67	0.37	0.60
		4806X	88.74	18.38	55.66	98.29	90.00	6.3	0.00	60.00		21870	1.0	5.00	0.53	0.60
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3
FUNKFARM	CONTROL	4806BTX	129.88	16.29	54.81	99.44	90.00	8.0	1.66	56.67		22240	1.0	2.00	0.47	0.93
		4806X	118.71	17.69	53.10	100.00	60.00	8.7	2.28	60.00		21497	3.3	3.67	0.60	1.13
	ECB1	4806BTX	122.67	17.04	53.89	100.00	90.00	5.3	2.22	60.00		22240	1.0	5.00	0.50	0.93
		4806X	121.18	17.75	53.60	100.00	73.33	6.7	0.55	50.00		22240	5.7	4.00	0.30	1.80

continued

Table 2B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS
FUNKFARM	ECB12	4806BTX	108.62	16.50	53.81	98.89	80.00	7.7	0.00	56.67		22240	1.0	8.00	0.40	0.93
		4806X	122.20	17.06	54.03	98.89	73.33	7.0	0.00	46.67		22240	6.0	8.33	1.80	2.67
	ECB2	4806BTX	123.32	16.45	54.24	98.89	90.00	8.0	0.55	56.67		22240	.	6.33	1.07	1.67
		4806X	122.77	16.82	54.52	99.44	83.33	6.7	1.66	53.33		22240	.	7.00	1.47	1.80
	PRTCT	4806BTX	117.06	16.42	54.52	98.89	93.33	7.3	0.00	56.67		22240	1.0	5.00	0.33	0.67
		4806X	118.94	17.52	53.96	100.00	70.00	6.7	5.55	56.67		22240	2.0	6.00	1.03	2.07
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3
GOEHNER	CONTROL	4806BTX	161.50	14.41	60.83	99.20	100.00	3.3	0.00		3.0	21520	1.0	.	.	.
		4806X	161.02	14.58	61.12	94.44	80.00	6.0	0.79		5.3	21520	1.7	.	.	.
	ECB1	4806BTX	146.32	14.70	61.54	99.20	100.00	3.3	0.00		3.0	21520	1.0	.	.	.
		4806X	159.98	16.25	59.41	98.41	100.00	3.0	0.00		3.7	21520	4.7	.	.	.
	ECB12	4806BTX	146.86	14.19	61.19	96.82	93.33	4.0	1.59		4.0	21520	1.0	.	.	.
		4806X	137.73	13.91	60.48	92.06	63.33	7.7	2.38		6.7	21520	3.3	.	.	.
	ECB2	4806BTX	156.39	14.73	60.41	99.20	100.00	2.0	0.79		2.7	21520		.	.	.
		4806X	165.54	15.72	59.20	97.61	96.67	3.0	0.79		2.7	21520		.	.	.
	PRTCT	4806BTX	157.17	15.49	59.49	100.00	100.00	2.7	0.00		3.0	21520	1.0	.	.	.
		4806X	158.71	16.59	59.77	99.19	100.00	2.3	0.00		3.3	21350	1.0	.	.	.
		N	3	3	3	3	3	3	3	0	3	3	3	0	0	0
LEROY	CONTROL	4806BTX	94.50	18.93	55.16	99.44	96.67	7.3	0.00	63.33		22240	1.0	5.00	0.83	0.67
		4806X	95.81	20.10	54.10	99.44	90.00	7.7	0.55	63.33		22240	3.3	4.67	1.37	1.40
	ECB1	4806BTX	113.21	18.68	55.02	98.33	93.33	5.3	0.00	63.33		22240	1.0	4.33	0.40	0.27
		4806X	98.14	19.43	54.03	99.44	90.00	7.7	1.11	63.33		22240	6.7	5.33	0.90	0.47
	ECB12	4806BTX	90.57	17.47	55.80	98.89	86.67	7.3	0.00	56.67		22240	1.0	8.33	0.57	1.27
		4806X	69.07	17.91	55.02	98.89	63.33	9.0	1.67	43.33		22117	6.3	7.33	1.17	1.33
	ECB2	4806BTX	89.51	17.24	55.87	98.89	86.67	6.7	0.00	56.67		22240		7.00	0.97	1.40
		4806X	80.83	17.72	54.81	95.00	56.67	9.0	1.11	43.33		22240		9.00	1.77	2.07
	PRTCT	4806BTX	93.94	18.47	55.23	100.00	100.00	5.7	0.00	60.00		22240	1	5.00	0.20	0.13
		4806X	97.20	19.44	53.82	100.00	90.00	6.0	0.00	60.00		22240	1	7.00	0.50	0.47
		N	3	3	3	3	3	3	3	3	0	3	3	3	3	3

continued

Table 2B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS
MARION	CONTROL	4806BTX	137.33	14.71	61.04	98.19	46.67	8.0	0.64	20.67	4.7	28390	1.0			
		4806X	140.05	16.20	59.55	95.11	66.67	8.7	0.60	14.67	5.3	26940	4.3			
	ECB1	4806BTX	152.33	14.60	63.03	98.97	53.33	8.0	0.50	19.67	4.3	31457	1.0			
		4806X	138.02	15.68	59.98	95.19	56.67	8.3	0.00	17.00	4.7	29523	5.3			
	ECB12	4806BTX	154.95	14.28	62.25	93.39	46.67	7.3	0.00	20.67	6.3	27423	1.0			
		4806X	127.02	16.24	59.63	96.72	53.33	8.3	0.00	18.67	5.7	29680	4.3			
	ECB2	4806BTX	159.83	15.55	60.55	97.72	76.67	6.7	0.00	23.00	4.7	28233				
		4806X	135.36	15.90	59.55	94.68	63.33	8.7	0.00	15.67	6.7	26940				
	PRTCT	4806BTX	135.60	15.04	60.48	98.42	63.33	7.0	0.53	22.00	5.7	30003	1			
		4806X	147.92	16.91	60.48	96.21	56.67	8.0	0.53	22.00	6.7	30007	1			
		N	3	3	3	3	3	3	3	3	3	3	3	0	0	0
OWATONA	CONTROL	4806BTX	103.08	28.66	52.54		73.33	6.3	0.00				1.0			
		4806X	99.68	30.44	52.68		86.67	6.3	0.00				1.7			
	ECB1	4806BTX	117.70	27.01	55.59		90.00	4.3	0.00				1.0			
		4806X	106.78	27.89	55.37		93.33	6.3	0.00				6.7			
	ECB12	4806BTX	111.08	26.74	54.45		73.33	7.7	0.00				1.7			
		4806X	88.95	27.01	54.17		60.00	8.3	0.00				6.3			
	ECB2	4806BTX	105.33	28.93	52.32		90.00	6.7	0.00							
		4806X	106.47	30.57	52.96		93.33	7.3	0.67							
	PRTCT	4806BTX	109.46	27.48	52.40		86.67	7.0	0.00				1			
		4806X	115.97	31.52	52.26		100.00	6.0	0.00				1			
		N	3	3	3	0	3	3	3	0	0	0	3	0	0	0
ROCHELLE	CONTROL	4806BTX	153.56	22.12	56.23	99.42	100.00	4.0		50.00		24060	1.0			
		4806X	145.45	25.63	52.89	98.19	93.33	5.3		40.00		23507	1.0			
	ECB1	4806BTX	157.96	22.22	54.81	100.00	100.00	2.7		51.67		24060	1.0			
		4806X	146.36	24.86	53.67	95.40	73.33	3.0		48.33		23920	8.0			
	ECB12	4806BTX	143.72	22.07	56.15	98.85	96.67	4.0		48.33		24060	1.0			
		4806X	119.76	23.68	55.09	95.97	50.00	6.3		25.00		24060	8.0			

continued

Table 2B continued

LOCATION	TREAT	HYBRID	YIELD	MOIST	TSTWT	%ERCT	%PUSH	INTCT	%DEAR	%GRN	APRLR	FPOPN	ECB1	ECB2	INCHTNL	#HOLS
ROCHELLE	ECB2	4806BTX	152.30	23.49	54.88	98.85	93.33	3.7		58.33		24060				
		4806X	150.22	25.49	55.37	99.42	86.67	4.3		51.67		24060				
	PRTCT	4806BTX	157.08	22.53	55.16	100.00	96.67	2.3		65.00		23783	1			
		4806X	173.49	25.29	52.82	100.00	96.67	2.7		61.67		23920	1			
		N	3	3	3	3	3	3	0	3	0	3	3	0	0	0
SHELBVLE	CONTROL	4806BTX	140.71	21.56	57.78		100.00	2.3				18663	1.0			
		4806X	152.21	22.75	56.79		100.00	2.7				19910	1.0			
	ECB1	4806BTX	155.65	22.03	58.14		100.00	1.7				19493	1.0			
		4806X	132.37	24.16	56.37		98.61	5.7				18527	4.0			
	ECB12	4806BTX	147.07	21.49	57.79		100.00	2.7				19217	1.0			
		4806X	127.97	20.65	57.92		93.05	8.7				19910	3.7			
	ECB2	4806BTX	142.07	20.80	57.85		98.61	2.3				19910				
		4806X	129.32	21.91	57.64		98.52	5.7				19493				
	PRTCT	4806BTX	146.78	21.18	58.21		100.00	2.0				19357	1			
		4806X	142.98	24.28	56.79		100.00	2.7				19910	1			
		N	3	3	3	0	3	3	0	0	0	3	3	0	0	0
LSD(0.05) - A			40.87	2.94	2.61	6.80	46.52	4.42	4.07	19.58	5.13	1677	3.30	7.28	2.52	3.63
LSD(0.05) - B			32.21	2.68	2.45	5.26	35.99	3.63	3.34	19.17	3.12	1864	2.68	4.27	1.71	2.00

A = FOR COMPARING WITHIN LOCATION VERSION MEANS IN THE SAME TREATMENT.
B = FOR COMPARING WITHIN LOCATION VERSION MEANS IN DIFFERENT TREATMENTS.

TABLE 3. COMBINED LOCATION MEANS FOR ECB1 DAMAGE IN PESTICIDE VS BTGENE EXPERIMENT 1994

INFESTATION REGIME	TREATMENT	ECB1	% INFESTATION
ECB1	BTGENE	1.16	0.00
	DIPEL	2.83	8.33
	LORSBAN	3.00	5.00
	POUNCE	2.50	6.67
	UNTRTD	4.50	71.67
NATURAL	BTGENE	1.00	0.00
	DIPEL	1.00	3.33
	LORSBAN	1.67	5.00
	POUNCE	1.16	10.00
	UNTRTD	1.83	36.67
	N	6	6
LSD(0.05) - A		2.44	17.74
LSD(0.05) - B		2.53	42.30

A = FOR COMPARING TREATMENT MEANS IN THE SAME INFESTATIONS.
B = FOR COMPARING TREATMENT MEANS IN DIFFERENT INFESTATIONS.

TABLE 4. SUMMARY OF MEANS FOR VISUAL ECB1 RATING AND FOR PERCENT INFESTED PLANTS BY LOCATIONS

LOCATION	INFESTATION REGIME	TREATMENT	ECB1	% INFESTED
ARC FARM	ECB1	BTGENE	1.33	0.00
		DIPEL	2.67	6.67
		LORSBAN	2.67	6.67
		POUNCE	2.00	10.00
		UNTRTD	3.00	53.33
	NATURAL	BTGENE	1.00	0.00
		DIPEL	1.00	3.33
		LORSBAN	2.00	3.33
		POUNCE	1.33	20.00
		UNTRTD	1.33	53.33
		N =	3	3
FUNKFARM	ECB1	BTGENE	1.00	0.00
		DIPEL	3.00	10.00
		LORSBAN	3.33	3.33
		POUNCE	3.00	3.33
		UNTRTD	6.00	90.00
	NATURAL	BTGENE	1.00	0.00
		DIPEL	1.00	3.33
		LORSBAN	1.33	6.67
		POUNCE	1.00	0.00
		UNTRTD	2.33	20.00
		N =	3	3
LSD(0.05) - A			3.44	25.09
LSD(0.05) - B			3.58	59.83

A = FOR COMPARING TREATMENT MEANS IN THE SAME INFESTATIONS.
B = FOR COMPARING TREATMENT MEANS IN DIFFERENT INFESTATIONS.

Table 5. A Comparative Study Evaluating The Number of Live ECB1 Larvae Present on Ciba Seeds *Bt* Maize and Isogenic Controls Treated With Commonly Used Insecticides

4

ECBRAW3: NUMBER OF LAVRVAE PER PLANT, ANALYSIS
General Linear Models Procedure

T tests (LSD) for variable: LARVAE

NOTE: This test controls the type I comparisonwise error rate not the experimentwise error rate.

Alpha= 0.05 df= 75 MSE= 0.013073
Critical Value of T= 1.99
Least Significant Difference= 0.1636
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 3.875776

Means with the same letter are not significantly different.

T Grouping			Mean	N	TRTMENT
	A		0.40000	3	EF130575WG.5BR
B	A		0.35000	4	Condor5G10b
B	A	C	0.27500	8	Untreated
B	D	C	0.20000	4	EF130575WG1br
B	D	C	0.20000	4	ABG6149 6b
E	D	C	0.17500	4	EF130575WG.25br
E	D	C	0.17500	4	Whir15G5b
E F	D	C	0.15000	4	ABG6389 6b
E F	D	C	0.15000	4	XRM53184EC1br
E F	D	C	0.15000	4	TD2344.42EC.02br
E F	D	C	0.15000	4	Whir15G10b
E F	D	C	0.15000	4	TD2344.42EC.04br
E F	D	C	0.12500	4	XRM53184EC.5br
E F	D	C	0.12500	4	XRM53184EC.25br
E F	D		0.10000	3	TD2344.42EC.03BR
E F	D		0.10000	4	TD23482FM.75br
E F	D		0.07500	4	TD23482FM.5br
E F	D		0.07500	4	Dipel10G10b
E F	D		0.06667	3	KARATE1EC3.2BR
E F			0.02500	4	Btmaize
F			0.00000	4	Pounce1.5G.12b
F			0.00000	4	RH5992.125+br
F			0.00000	4	RH5992.25+br
F			0.00000	3	RH2485.0625+BR
F			0.00000	4	RH2485.125+br
F			0.00000	4	M-Peril6b

Table 6. A Comparative Study Evaluating the Number of Cavities Per Plant As A Result Of ECB1 Infestation Of Ciba Seeds *Bt* Maize and Isogenic Controls Treated With Commonly Used Insecticides.

Alpha= 0.05 df= 75 MSE= 0.058605
Critical Value of T= 1.99
Least Significant Difference= 0.3464
WARNING: Cell sizes are not equal.
Harmonic Mean of cell sizes= 3.875776

Means with the same letter are not significantly different.

T Grouping						Mean	N	TRTMENT
			A			0.9125	8	Untreated
			A			0.9000	4	Whirl5G5b
	B		A			0.8500	4	Condor5G10b
	B		A	C		0.7667	3	EF130575WG.5BR
	B	D	A	C		0.7250	4	TD2344.42EC.02br
E	B	D	A	C		0.6250	4	ABG6389 6b
E	B	D	A	C		0.6250	4	TD23482FM.75br
E	B	D	A	C	F	0.6000	3	XRM53184EC.25BR
E	B	D		C	F	0.5500	4	Dipel10G10b
E	B	D		C	F	0.5250	4	XRM53184EC.5br
E	B	D		C	F	0.5250	4	ABG6149 6b
E	B	D		C	F	0.5250	4	EF130575WG.25br
E		D	G	C	F	0.5000	4	XRM53184EC1br
E		D	G	C	F	0.4333	3	TD2344.42EC.03BR
E		D	G	C	F	0.4250	4	TD2344.42EC.04br
E		D	G		F	0.4000	4	Pounce1.5G.12b
E	H		G		F	0.3500	4	Whirl5G10b
E	H		G		F	0.3500	4	TD23482FM.5br
E	H		G		F	0.3250	4	M-Peril6b
	H		G		F	0.2750	4	Karate1EC3.2br
	H		G		F	0.2750	4	EF130575WG1br
	H		G			0.1750	4	RH5992.125+br
	H					0.0500	4	RH5992.25+br
	H					0.0333	3	RH2485.0625+BR
	H					0.0250	4	Bt maize
	H					0.0250	4	RH2485.125+br

ECBBROW2. % OF PLANTS INFESTED MEAN & STD

			A		63.75	8	Untreated
	B		A		57.50	4	Condor5G10b
	B		A	C	55.00	4	Whir15G5b
	B	D	A	C	50.00	3	EF130575WG.5br
E	B	D	A	C	45.00	4	TD2344.42EC.02br
E	B	D	A	C	42.50	4	XRM53184EC1br
E	B	D	A	C	42.50	4	XRM53184EC.5br
E	B	D		C	40.00	4	ABG6389 6b
E	B	D		C	40.00	4	Dipel10G10b
E	B	D		C	40.00	4	TD23482FM.75br
E	B	D	F	C	37.50	4	EF130575WG.25br
E	B	D	F	C	36.67	3	XRM53184EC.25br
E		D	F	C	32.50	4	EF130575WG1br
E		D	F		30.00	4	TD23482FM.5br
E		D	F		30.00	4	ABG6149 6b
E	G	D	F		27.50	4	Pounce1.5G.12b
E	G	D	F		27.50	4	TD2344.42EC.04br
E	G	D	F	H	26.67	3	TD2344.42EC.03br
E	G	I	F	H	25.00	4	Karate1EC3.2br
E	G	I	F	H	25.00	4	Whir15G10b
E	G	I	F	H	25.00	4	M-Peril6b
	G	I	F	H	15.00	4	RH5992.125+br
	G	I		H	05.00	4	RH5992.25+br
		I		H	03.33	3	RH2485.0625+br
		I			02.50	4	Btmaize
		I			02.50	4	RH2485.125+br

Table 7. A Comparative Study Of The Number Of Plants That Exhibit ECB1 Infestation Following Artificial Infestation With ECB1 Egg Masses of Ciba Seeds *Bt* Maize And Isogenic Controls Treated With Commonly Used Insecticides

TABLE 8. TREATMENT MEANS FOR BTGENE VS PESTICIDE STUDY 1994

INFREGM	TREATMENT	YIELD	MOIST	TSTWT	BROKN	DREAR	PEPSH	INTCT	FPOPN	ECB2RT	TNWHPLT	NUMHOLS
ECB12	BTGENE	144.38	18.82	55.91	0.00	0.00	91.67	5.00	23740	3.15	0.28	0.52
	DIPEL	139.54	19.51	55.73	0.00	0.00	95.00	6.84	23885	6.33	0.58	0.77
	LORSBAN	153.48	19.88	55.69	0.17	0.00	96.67	4.33	23958	4.75	0.20	0.45
	POUNCE	148.82	20.10	56.05	0.00	0.00	96.67	4.84	23522	2.50	0.03	0.13
	UNTRTD	139.34	19.16	56.30	0.17	0.00	76.67	7.34	23595	7.45	1.15	2.27
ECB2	BTGENE	136.35	18.74	57.08	0.00	0.00	96.67	5.17	23958	2.55	0.23	0.27
	DIPEL	148.11	19.71	55.98	0.17	0.00	100.00	5.67	23740	5.47	0.33	0.42
	LORSBAN	143.27	20.01	54.85	0.00	0.00	93.34	5.67	23958	5.67	0.44	0.55
	POUNCE	143.56	20.04	55.09	0.17	0.00	93.34	5.00	23014	2.95	0.30	0.32
	UNTRTD	135.54	19.93	55.13	0.00	0.17	91.67	7.17	23305	6.22	0.45	0.57
NATRL	BTGENE	128.93	18.52	57.79	0.00	0.00	90.00	5.67	23087	3.55	0.29	0.43
	DIPEL	132.28	20.25	54.70	0.17	0.00	85.00	5.67	23014	4.60	0.10	0.36
	LORSBAN	143.25	20.35	54.66	0.00	0.00	98.34	5.50	23668	4.40	0.10	0.23
	POUNCE	154.67	20.01	55.23	0.00	0.00	95.00	5.50	23305	3.65	0.04	0.08
	UNTRTD	124.09	20.02	55.05	0.00	0.00	80.00	7.33	23522	5.90	0.47	0.80
	N	6	6	6	6	6	6	6	6	6	6	6
LSD(0.05) - A		21.86	1.24	1.62	0.49	0.17	23.04	4.6	3741	5.50	0.78	0.93
LSD(0.05) - B		21.94	1.12	1.47	0.45	0.16	23.65	4.1	3310	5.06	0.70	0.95

A = FOR COMPARING TREATMENT MEANS IN THE SAME INFESTATIONS.
B = FOR COMPARING TREATMENT MEANS IN DIFFERENT INFESTATIONS.

TABLE 9. WITHIN LOCATION AND TREATMENT MEANS FOR THE BTGENE VS PESTICIDE FORMULATIONS EXPERIMENT SECOND BROOD EXPERIMENT 1994

LOCATION	INFREGM	TREATMENT	YIELD	MOIST	TSTWT	BROKN	DREAR	PEPSH	INTCT	FPOPN	ECB2RT	TNWHPLT	NUMHOLS
ARC_FARM	ECB12	BTGENE	165.09	15.88	61.04	0.00	0.00	93.33	6.7	24974	2.50	0.48	0.83
		DIPEL	152.81	16.59	60.90	0.00	0.00	93.33	7.7	23522	4.20	0.62	0.80
		LORSBAN	177.55	17.17	59.84	0.33	0.00	93.33	6.3	23522	1.90	0.10	0.33
		POUNCE	170.85	17.08	61.40	0.00	0.00	96.67	7.0	22651	1.60	0.03	0.10
		UNTRTD	157.90	16.58	60.83	0.00	0.00	76.67	8.0	23522	5.90	1.00	2.30
	ECB2	BTGENE	162.33	15.87	61.54	0.00	0.00	93.33	7.7	24684	2.10	0.23	0.20
		DIPEL	171.64	16.81	60.76	0.00	0.00	100.00	7.3	23232	2.40	0.07	0.10
		LORSBAN	166.26	17.40	59.56	0.00	0.00	86.67	6.3	23522	3.40	0.28	0.33
		POUNCE	153.72	17.10	60.12	0.33	0.00	86.67	6.3	21635	1.50	0.23	0.13
		UNTRTD	158.30	17.38	59.91	0.00	0.33	96.67	7.3	22216	3.30	0.19	0.13
	NATRL	BTGENE	141.70	15.69	62.89	0.00	0.00	96.67	7.7	22506	2.00	0.35	0.40
		DIPEL	143.13	17.74	58.99	0.00	0.00	100.00	7.0	22361	2.20	0.03	0.20
		LORSBAN	166.54	17.65	59.41	0.00	0.00	96.67	6.7	22942	1.90	0.03	0.07
		POUNCE	174.55	16.87	60.41	0.00	0.00	96.67	7.3	22506	2.20	0.05	0.03
		UNTRTD	130.38	17.47	60.12	0.00	0.00	83.33	7.3	22651	2.80	0.22	0.27
		N	3	3	3	3	3	3	3	3	3	3	3
FUNKFARM	ECB12	BTGENE	123.66	21.75	50.77	0.00	0.00	90.00	3.3	22506	3.80	0.08	0.20
		DIPEL	126.26	22.43	50.55	0.00	0.00	96.67	6.0	24248	8.47	0.53	0.73
		LORSBAN	129.40	22.58	51.54	0.00	0.00	100.00	2.3	24394	7.60	0.30	0.57
		POUNCE	126.79	23.11	50.69	0.00	0.00	96.67	2.7	24394	3.40	0.03	0.17
		UNTRTD	120.77	21.74	51.76	0.33	0.00	76.67	6.7	23668	9.00	1.30	2.23
	ECB2	BTGENE	110.36	21.60	52.61	0.00	0.00	100.00	2.7	23232	3.00	0.23	0.33
		DIPEL	124.57	22.60	51.19	0.33	0.00	100.00	4.0	24248	8.53	0.60	0.73
		LORSBAN	120.28	22.61	50.13	0.00	0.00	100.00	5.0	24394	7.93	0.60	0.77
		POUNCE	133.39	22.97	50.06	0.00	0.00	100.00	3.7	24394	4.40	0.37	0.50
		UNTRTD	112.78	22.47	50.34	0.00	0.00	86.67	7.0	24394	9.13	0.72	1.00
	NATRL	BTGENE	116.15	21.34	52.68	0.00	0.00	83.33	3.7	23668	5.10	0.23	0.47
		DIPEL	121.43	22.75	50.41	0.33	0.00	70.00	4.3	23668	7.00	0.16	0.52
		LORSBAN	119.96	23.05	49.91	0.00	0.00	100.00	4.3	24394	6.90	0.17	0.40
		POUNCE	134.79	23.14	50.05	0.00	0.00	93.33	3.7	24103	5.10	0.03	0.13
		UNTRTD	117.79	22.56	49.98	0.00	0.00	76.67	7.3	24394	9.00	0.73	1.33
		N	3	3	3	3	3	3	3	3	3	3	3
LSD(0.05) - A			30.91	1.76	2.29	0.70	0.24	32.59	6.52	5291	7.78	1.10	1.32
LSD(0.05) - B			31.03	1.59	2.08	0.63	0.22	33.45	5.87	4680	7.16	0.99	1.35

A = FOR COMPARING TREATMENT MEANS IN THE SAME INFESTATIONS.
B = FOR COMPARING TREATMENT MEANS IN DIFFERENT INFESTATIONS.

TABLE 10. PERFORMANCE OF *BT* AND CONTROL HYBRIDS IN PLOTS NATURALLY INFESTED WITH ECB IN 1994

State	County	Cooperator	Treatment	Yield (bu/a)	Moisture (%)	Test Wt (lb/bu)	Entry Holes (#/plant)	Dropped Ears (#)	Population (000/acre)	Erect (%)	E Push (%)
CO	Yuma	Goeglein	Bt+	54.5	22.20	53.5	0.3	1.0	25.20	95.0	91.0
			Bt-	48.9	21.20	53.3	1.6	2.0	31.60	88.0	74.0
			Difference	5.6	1.00	0.2	-1.3	-1.0	-6.40	7.0	17.0
			n=	1.0	1.00	1.0	1.0	1.0	1.00	1.0	1.0
			MEAN	**5.6**	**1.00**	**0.2**	**-1.3**	**-1.0**	**-6.40**	**7.0**	**17.0**
IA	Bremer	Kratchmer	Bt+	216.0	24.30	52.8	5.0	0.0	21.00		
			Bt-	184.0	26.20	52.5	10.0	2.0	21.00		
			Difference	32.0	-1.90	0.3	-5.0	-2.0	0.00	0.0	0.0
IA	O'Brien	Mugge	Bt+	120.0	22.00	53.4	1.8	0.0	28.20	99.2	97.9
			Bt-	110.5	21.10	55.0	4.3	0.0	29.00	96.4	81.4
			Difference	9.5	0.90	-1.6	-2.5	0.0	-0.80	2.8	16.5
IA	Washington	Schaffer	Bt+	157.0	20.50	57.0	3.0	1.0	23.30		
			Bt-	152.0	22.20	55.0	11.0	0.0	24.70		
			Difference	5.0	-1.70	2.0	-8.0	1.0	-1.40	0.0	0.0
			n=	3.0	3.00	3.0	3.0	3.0	3.00	1.0	1.0
			MEAN	**15.5**	**-0.90**	**0.2**	**-5.2**	**-0.3**	**-0.73**	**2.8**	**16.5**
IL	Carroll	Smith	Bt+	174.8			1.3		19.70	100.0	79.1
			Bt-	153.5			6.1		17.30	96.3	53.9
			Difference	21.3	0.00	0.0	-4.8	0.0	2.40	3.7	25.2
IL	Cass	Merwin	Bt+	46.2	22.00	59.0	0.0	0.0	21.80	100.0	100.0
			Bt-	41.5	23.00	59.0	0.0	0.0	19.80	100.0	98.9
			Difference	4.7	-1.00	0.0	0.0	0.0	2.00	0.0	1.1
IL	Gallatin	Downen	Bt+	104.0	21.00	63.0	0.0	0.0	17.00	98.0	14.0
			Bt-	114.0	20.00	63.0	1.0	0.0	17.40	99.0	23.0
			Difference	-10.0	1.00	0.0	-1.0	0.0	-0.40	-1.0	-9.0
IL	Henry	Corkill	Bt+	164.6	18.30	61.5	0.6	0.0	24.80	100.0	97.0
			Bt-	145.9	20.50	58.8	3.8	0.0	26.30	97.1	91.6
			Difference	18.7	-2.20	2.7	-3.2	0.0	-1.50	2.9	5.4
IL	Kane	Drendel	Bt+	171.9	23.70	53.3	1.4		23.30	97.1	66.1
			Bt-	143.4	26.50	52.7	4.7		19.70	96.1	46.1
			Difference	28.5	-2.80	0.6	-3.3	0.0	3.60	1.0	20.0

continued

Table 10 continued

State	County	Cooperator	Treatment	Yield (bu/a)	Moisture (%)	Test Wt (lb/bu)	Entry Holes (#/plant)	Dropped Ears (#)	Population (000/acre)	Erect (%)	E Push (%)
IL	Marion	Hester	Bt+	94.5	18.00	64.2	2.5	0.0	22.40	90.8	78.3
			Bt-	75.3	18.30	63.8	4.5	1.8	23.20	91.3	73.4
			Difference	19.2	-0.30	0.4	-2.0	-1.8	-0.80	-0.5	4.9
IL	Wayne	Robbins	Bt+	95.2	38.90						
			Bt-	87.2	40.20						
			Difference	8.0	-1.30	0.0	0.0	0.0	0.00	0.0	0.0
			n=	7.0	6.00	5.0	6.0	4.0	6.00	6.0	6.0
			MEAN	**12.9**	**-1.10**	**0.7**	**-2.4**	**-0.5**	**0.88**	**1.0**	**7.9**
IN	Boone	Poole	Bt+	178.9	24.90	57.4	0.5	0.0	21.20	96.4	94.5
			Bt-	150.8	26.70	56.3	1.7	2.6	20.00	96.1	94.1
			Difference	28.1	-1.80	1.1	-1.2	-2.6	1.20	0.3	0.4
IN	Huntington	Platt	Bt+	192.4	17.90	60.0	0.1	0.0	25.80	99.4	98.7
			Bt-	185.5	18.80	59.8	1.3	2.7	25.20	97.9	96.3
			Difference	6.9	-0.90	0.2	-1.3	-2.7	0.60	1.5	2.4
IN	Montgomery	Traylor	Bt+	129.0	26.10		0.1	0.0	15.80	100.0	100.0
			Bt-	129.9	27.10		0.3	0.0	17.80	100.0	100.0
			Difference	-0.9	-1.00	0.0	-0.2	0.0	-2.00	0.0	0.0
IN	Rush	Miller	Bt+	69.2	24.50		0.0	0.0	22.20	99.0	45.8
			Bt-	62.1	24.40		0.1	1.1	20.20	99.0	38.8
			Difference	7.1	0.10	0.0	-0.1	-1.1	2.00	0.0	7.0
			n=	4.0	4.00	2.0	4.0	4.0	4.00	4.0	4.0
			MEAN	**10.3**	**-0.68**	**1.1**	**-0.4**	**-0.9**	**0.30**	**0.1**	**1.9**
KY	Hopkins	Stanley	Bt+	167.0	17.10	64.5	0.0	0.0	21.50	88.1	71.3
			Bt-	157.0	16.50	65.5	0.0	0.0	26.00	88.4	67.7
			Difference	10.0	0.60	-1.0	0.0	0.0	-4.50	-0.3	3.6
			n=	1.0	1.00	1.0	1.0	1.0	1.00	1.0	1.0
			MEAN	**10.0**	**0.60**	**-1.0**	**0.0**	**0.0**	**-4.50**	**-0.3**	**3.6**
MI	Hillsdale	Lewis	Bt+	137.4	22.70	55.0	0.5	0.0	18.00	97.7	93.1
			Bt-	118.9	23.10	55.0	1.3	2.7	16.00	93.4	75.0
			Difference	18.5	-0.40	0.0	-0.8	-2.7	2.00	4.3	18.1
			n=	1.0	1.00	1.0	1.0	1.0	1.00	1.0	1.0
			MEAN	**18.5**	**-0.40**	**0.0**	**-0.8**	**-2.7**	**2.00**	**4.3**	**18.1**

continued

Table 10 continued

State	County	Cooperator	Treatment	Yield (bu/a)	Moisture (%)	Test Wt (lb/bu)	Entry Holes (#/plant)	Dropped Ears (#)	Population (000/acre)	Erect (%)	E Push (%)
MN	Jackson	Gohr	Bt+	88.1	16.60	53.0	1.1	0.0	20.80	100.0	
			Bt-	68.7	17.10	53.0	1.2	0.0	15.80	100.0	
			Difference	19.4	-0.50	0.0	-0.1	0.0	5.00	0.0	0.0
MN	Jackson	Ploehn	Bt+	175.6	19.20	52.2	0.9	0.0	19.00	97.9	94.9
			Bt-	153.9	20.60	52.2	1.5	0.0	20.40	96.2	84.4
			Difference	21.7	-1.40	0.0	-0.6	0.0	-1.40	1.7	10.5
MN	Pipestone	Veldhuizen	Bt+	135.2	23.60	50.4	0.3	0.0	24.80	100.0	91.1
			Bt-	111.8	25.50	49.2	0.9	0.0	22.20	99.0	80.4
			Difference	23.4	-1.90	1.2	-0.6	0.0	2.60	1.0	10.7
MN	Redwood	Swanson	Bt+	184.7	18.80	56.0	0.5	0.0	22.00	100.0	96.4
			Bt-	183.5	19.20	55.2	1.9	0.0	22.20	100.0	95.2
			Difference	1.2	-0.40	0.8	-1.4	0.0	-0.20	0.0	1.2
			n=	4.0	4.00	4.0	4.0	4.0	4.00	4.0	3.0
			MEAN	**16.4**	**-1.05**	**0.5**	**-0.7**	**0.0**	**1.50**	**0.7**	**7.5**
MO	Atchison	Mulvania	Bt+	139.4	17.60	62.3	0.0	0.0	17.80	95.7	95.7
			Bt-	143.8	17.60	62.0	3.3	2.4	20.30	94.9	86.3
			Difference	-4.4	0.00	0.3	-3.3	-2.4	-2.50	0.8	9.4
MO	Lafayette	Rhodes	Bt+	184.4	23.00		0.0	0.0	19.50	100.0	100.0
			Bt-	171.6	23.00		0.0	0.0	19.50	100.0	100.0
			Difference	12.8	0.00	0.0	0.0	0.0	0.00	0.0	0.0
MO	Macon	Moots	Bt+	107.5	26.50	59.0					
			Bt-	109.7	25.50	58.5					
			Difference	-2.2	1.00	0.5	0.0	0.0	0.00	0.0	0.0
MO	Saline	Kirchhoff	Bt+	157.0	16.80	64.0	0.0	0.0	21.30	100.0	100.0
			Bt-	129.4	17.00	63.3	1.7	0.0	19.00	98.7	80.8
			Difference	27.6	-0.20	0.7	-1.7	0.0	2.30	1.3	19.2
			n=	4.0	4.00	3.0	3.0	3.0	3.00	3.0	3.0
			MEAN	**8.5**	**0.20**	**0.5**	**-1.7**	**-0.8**	**-0.07**	**0.7**	**9.5**
NE	Boone	Nelson	Bt+	140.8	18.00	56.0	1.9	0.9	21.00	100.0	85.0
			Bt-	108.3	18.20	56.4	5.5	2.2	20.00	89.0	57.0
			Difference	32.5	-0.20	-0.4	-3.6	-1.3	1.00	11.0	28.0

continued

Table 10 continued

State	County	Cooperator	Treatment	Yield (bu/a)	Moisture (%)	Test Wt (lb/bu)	Entry Holes (#/plant)	Dropped Ears (#)	Population (000/acre)	Erect (%)	E Push (%)
NE	Cass	Rolofsan	Bt+	143.1	22.60	55.7	1.1	0.0	16.80	100.0	93.0
			Bt-	136.7	22.60	55.0	3.9	3.6	16.40	94.0	69.0
			Difference	6.4	0.00	0.7	-2.8	-3.6	0.40	6.0	24.0
NE	Cedar	Lute	Bt+	137.8	19.10	57.9	1.2	3.3	21.00	97.0	88.0
			Bt-	116.5	20.40	56.1	5.5	6.7	21.60	85.0	55.0
			Difference	21.3	-1.30	1.8	-4.3	-3.4	-0.60	12.0	33.0
NE	Chase	Bauerle	Bt+	123.9	13.10	60.3	0.5	1.3	33.80	96.0	91.0
			Bt-	85.6	14.40	59.7	4.6	7.4	31.00	88.0	80.0
			Difference	38.3	-1.30	0.6	-4.1	-6.1	2.80	8.0	11.0
NE	Dakota	Rohde	Bt+	193.3	19.80	55.4	0.5	1.6	27.20	96.0	91.0
			Bt-	176.0	20.40	54.1	3.5	2.9	25.80	94.0	81.0
			Difference	17.3	-0.60	1.3	-3.0	-1.3	1.40	2.0	10.0
NE	Dawson	Tauberheim	Bt+	141.3	19.80	60.4	1.5	7.0	23.60	91.0	74.0
			Bt-	104.9	19.70	59.5	4.0	18.5	27.40	80.0	53.0
			Difference	36.4	0.10	0.9	-2.5	-11.5	-3.80	11.0	21.0
NE	Kearney	Nelson	Bt+	131.8	19.10	57.8	1.5	2.7	22.80	97.0	76.0
			Bt-	112.6	19.90	54.4	3.2	4.1	23.40	96.0	70.0
			Difference	19.2	-0.80	3.4	-1.7	-1.4	-0.60	1.0	6.0
NE	Madison	Praurver	Bt+	136.5	27.60	55.8	2.2	3.0	22.00	97.0	86.0
			Bt-	113.2	31.40	54.6	5.3	2.2	20.40	92.0	71.0
			Difference	23.3	-3.80	1.2	-3.1	0.8	1.60	5.0	15.0
NE	Nemaha	Gerdes	Bt+	24.6	18.10	56.0	0.1	0.0	12.20	90.0	81.0
			Bt-	34.6	19.90	55.3	0.5	2.2	12.60	95.0	87.0
			Difference	-10.0	-1.80	0.7	-0.4	-2.2	-0.40	-5.0	-6.0
NE	Pierce	Stech	Bt+	159.9	22.20	56.9	1.4	2.9	22.80	94.0	90.0
			Bt-	146.6	22.50	56.0	4.1	8.7	24.20	88.0	63.0
			Difference	13.3	-0.30	0.9	-2.7	-5.8	-1.40	6.0	27.0
NE	Platte	Bender	Bt+	85.2	20.60	56.1	1.1	3.8	16.80	90.0	82.0
			Bt-	63.9	21.70	55.1	3.7	8.7	15.80	91.0	66.0
			Difference	21.3	-1.10	1.0	-2.6	-4.9	1.00	-1.0	16.0
			n=	11.0	11.00	11.0	11.0	11.0	11.00	11.0	11.0
			MEAN	**19.9**	**-1.01**	**1.1**	**-2.8**	**-3.7**	**0.13**	**5.1**	**16.8**

continued

Table 10 continued

State	County	Cooperator	Treatment	Yield (bu/a)	Moisture (%)	Test Wt (lb/bu)	Entry Holes (#/plant)	Dropped Ears (#)	Population (000/acre)	Erect (%)	E Push (%)
OH	Shelby	Roeth	Bt+	153.6	29.80		0.0	0.0	19.40	100.0	100.0
			Bt-	162.5	30.30		0.4	0.0	22.80	100.0	97.4
			Difference	-8.9	-0.50	0.0	-0.4	0.0	-3.40	0.0	2.6
OH	Tuscawaras	Jones	Bt+						25.00		
			Bt-						24.50		
			Difference	0.0	0.00	0.0	0.0	0.0	0.50	0.0	0.0
OH	VanWert	Eddy	Bt+	129.0	17.70	59.4	0.1		18.00	100.0	98.0
			Bt-	122.2	17.80	59.2	0.9		18.00	98.9	89.4
			Difference	6.8	-0.10	0.2	-0.8	0.0	0.00	1.1	8.6
			n=	2.0	2.00	1.0	2.0	1.0	3.00	2.0	2.0
			MEAN	**-1.1**	**-0.30**	**0.2**	**-0.6**	**0.0**	**-1.13**	**0.5**	**5.6**
SD	Beadle	Baruth	Bt+	103.4	16.90	58.2	1.1	0.0	17.00	96.4	95.3
			Bt-	116.4	16.90	57.6	2.1	1.2	19.80	95.9	89.8
			Difference	-13.0	0.00	0.6	-1.0	-1.2	-2.80	0.5	5.5
SD	Sully	Tracy	Bt+	140.3	22.60	54.2	1.8	0.0	20.60	97.9	
			Bt-	97.6	21.00	53.2	4.1	0.0	16.60	86.8	
			Difference	42.7	1.60	1.0	-2.3	0.0	4.00	11.1	0.0
SD	Turner	Bones	Bt+	189.7	27.00	52.8	0.5	1.0	20.80	98.0	95.0
			Bt-	183.5	28.80	52.1	1.3	0.9	22.20	97.0	82.0
			Difference	6.2	-1.80	0.7	-0.8	0.1	-1.40	1.0	13.0
			n=	3.0	3.00	3.0	3.0	3.0	3.00	3.0	2.0
			MEAN	**12.0**	**-0.07**	**0.8**	**-1.4**	**-0.4**	**-0.07**	**4.2**	**9.3**
Overall Mean Difference				**13.78**	**-0.68**	**0.68**	**-2.01**	**-1.59**	**0.00**	**2.60**	**10.81**
Probability of > Paired T Value=				1.44E-07	4.62E-04	1.58E-04	1.15E-08	4.45E-04	9.95E-01	2.99E-04	2.62E-07
Significance of the Paired T-Test=				**	**	**	**	**	NS	**	**

TABLE 11. 1994 *Bt* Maize Field Sites*

State	# of Sites	Acreage	# of Plants
California	1	0.1	1,800
Colorado	2	0.2	3,600
Florida	1	4.0	98,000
Hawaii	1	6.0	138,000
Illinois	9	0.9	16,200
Indiana	4	0.4	7,200
Iowa	6	0.6	10,800
Kansas	1	0.1	1,800
Kentucky	1	0.1	1,800
Michigan	1	0.1	1,800
Minnesota	4	0.4	7,200
Massachusetts	1	0.1	1,800
Missouri	5	0.5	9,000
Nebraska	11	1.1	19,800
North Carolina	2	0.2	3,600
Ohio	3	0.3	5,400
Pennsylvania	1	0.1	1,800
South Dakota	1	0.1	1,800
Total	55	15.3	331,400

*Field tests were conducted under USDA permits #94-056-06N and #94-076-10N, and EPA Experimental Use Permit #66736-EUP-1.

TABLE 12: Field Performance of Seven *Bt* Hybrids in 1994

HYBRID*	BUAC	NUMEAR	H_2O	WETEWT	DRYEWT	TSTWT	EGROTH	EVIGOR	POL	SILK	EARHT	PLTHT	EARLT	EARDIA
BtW1	143.37	61.11	14.15	30.40	21.62	60.59	2.00	3.25	55.50	54.00	38.42	88.17	7.57	2.06
IC	146.50	55.00	14.59	27.77	19.80	59.64	3.13	4.25	56.00	55.00	39.67	89.08	7.62	2.08
LSD(0.05)	6.76	3.51	NS^	1.41	1.05	NS	NS	NS	NS	NS	NS	1.96	0.12	0.03
#Locations	9	9	9	9	9	2	2	1	1	1	6	6	9	9
BtW2	150.22	60.44	14.49	31.20	22.14	58.89	2.63	4.00	56.50	55.50	40.17	91.08	7.70	2.08
IC	146.50	55.00	14.59	27.77	19.80	59.64	3.13	4.25	56.00	55.00	39.67	89.08	7.62	2.08
LSD(0.05)	6.76	3.51	NS	1.41	1.05	NS	NS	NS	NS	NS	NS	1.96	0.12	0.03
#Locations	9	9	9	9	9	2	2	1	1	1	6	6	9	9
BtX1	138.87	60.33	15.18	27.93	18.90	58.26	3.50	5.50	55.25	56.25	35.29	88.57	7.07	2.07
IC	147.75	60.28	15.58	29.14	20.11	56.73	2.92	5.00	55.50	55.50	38.79	88.86	7.00	2.16
LSD(0.05)	7.19	3.94	NS	1.76	1.07	NS	0.6	NS	NS	NS	2.21	NS	0.12	0.02
#Locations	10	9	10	9	9	2	3	1	2	2	7	7	9	9
BtX2	142.59	53.72	15.61	27.44	18.34	57.40	3.92	4.50	56.25	56.75	36.00	88.93	7.21	2.12
IC	147.75	60.28	15.58	29.14	20.11	56.73	2.92	5.00	55.50	55.05	38.79	88.86	7.00	2.16
LSD(0.05)	7.19	3.94	NS	1.76	1.07	NS	0.6	NS	NS	NS	2.21	NS	0.12	0.02
#Locations	10	9	10	9	9	2	3	1	2	2	7	7	9	9
BtY1	145.68	53.89	16.99	30.91	21.34	56.15	2.67	3.00	55.25	56.25	36.93	89.79	7.36	2.28
IC	143.37	53.56	17.65	30.70	20.99	56.70	3.00	3.00	55.25	55.75	39.57	89.14	7.16	2.31
LSD(0.05)	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
#Locations	10	9	10	9	9	2	3	1	2	2	7	7	9	9
BtZ1	147.82	44.57	19.22	28.71	19.26	55.05	4.00	4.75	58.50	58.00	42.20	89.10	7.17	2.58
IC	149.44	45.14	20.16	28.49	19.38	54.65	5.00	4.50	59.50	58.00	40.60	87.50	7.28	2.56
LSD(0.05)	NS	NS	NS	NS	NS	NS	NS	NS	0.88	NS	2.44	NS	NS	NS
#Locations	8	7	8	7	7	2	2	1	2	2	5	5	7	7
BtZ2	148.91	45.07	19.88	28.74	19.33	54.81	4.38	5.25	59.25	58.50	39.60	86.50	7.25	2.59
IC	149.44	45.14	20.16	28.49	19.38	54.65	5.00	4.50	59.50	58.00	40.60	87.50	7.28	2.56
LSD(0.05)	NS	NS	NS	NS	NS	NS	NS	NS	0.88	NS	2.44	NS	NS	NS
#Locations	8	7	8	7	7	2	2	1	2	2	5	5	7	7

*Bt designation refers to *Bt* transgenic hybrids derived from Event 176; IC is the near isogenic control.
^NS = not significant;
BUAC, bushels/acre; NUMEAR, # of ears harvested; H_2O, grain moisture; WETEWT, wet ear weight; DRYEWT, dry ear weight; TSTWT, test weight; EGROTH, early growth; EVIGOR, early vigor; POL, Days from planting to 50% pollen shed; SILK, Days from planting to 50% silking; EARHT, ear height; PLTHT, plant height; EARLT, average ear length of 10 random ears; EARDIA, average ear diameter of 10 random ears

TABLE 13. Disease Incidence Comparisons Between Bt maize hybrids and Isogenic Control Germplasm Observed in 1994 Field Trials.[1]

Disease[2]	Agent	Remarks
Eyespot	*Kabatiella zeae*	Equal incidence
Stewart's wilt	Various bacterial species	High incidence across both control and transgenic lines
Common rust	*Puccinia* sp.	Low incidence across both control and transgenic lines
Gray leaf spot	*Cercospora* sp.	High incidence across both control and transgenic lines
Anthracnose stalk rot	*Colletotrichum* sp.	Higher incidence on control, non-*Bt* plants[3]
Fusarium stalk rot	*Fusarium* sp.	Higher incidence on control, non-*Bt* plants[3]

[1]Based upon visual inspections of field sites throughout the growing season by professional plant breeders and agronomists.
[2]In addition to these diseases, the following diseases were inspected for but were not observed in these field trials: Southern rust, Goss's wilt, Northern corn leaf blight, Southern corn leaf blight, and Ear rot complexes.
[3]These fungal diseases had advanced from the immediate vicinity of the bore hole caused by the feeding of the European corn borer (ECB). Damage to stalks by ECB feeding provide fungal spores access to wounds needed for infection initiation.

TABLE 14. AFLATOXIN ANALYSIS OF KERNELS FROM CONTROL AND *BT* MAIZE PLANTS

Genotype[1]	Aflatoxin	ppb									
		Nontransgenic					Transgenic				
		Replicate Samples[2]					Replicate Samples[2]				
		1	2	3	4	5	1	2	3	4	5
CG00526	B_1	0	0	0	0	3.4	0	0	14.7	0	0
	B_2	0	0	0	0	0.5	0	0	1.3	0	0
	G_1	0	0	0	0	0	0	0	0	0	0
	G_2	0	0	0	0	0	0	0	0	0	0
CG00554 x CG00526	B_1	0	2.0	0	0	0	0	0	0	0	0
	B_2	0	0	0	0	0	0	0	0	0	0
	G_1	0	0	0	0	0	0	0	0	0	0
	G_2	0	0	0	0	0	0	0	0	0	0
CG00637 x CG00526	B_1	94.2	0	13.4	139.0	3.5	0	13.4	221.5	3.0	2.9
	B_2	1.8	0	0	5.0	0.5	0	0	18.5	0.4	0.5
	G_1	0	0	0	0	0	0	0	0	0	0
	G_2	0	0	0	0	0	0	0	0	0	0
CG00684 x CG00526	B_1	0	0	35.0	0	3.4	157.0	0	2.6	0	0
	B_2	0	0	1.0	0	0	9.0	0	0	0	0
	G_1	0	0	0	0	0	0	0	0	0	0
	G_2	0	0	0	0	0	0	0	0	0	0

[1]"CG" numbers designate proprietary Ciba Seeds inbred lines. See text for complete description of maize genotypes employed in this study.

[2]Five 100 g samples of kernels of each genotype was taken from a pooled sample representing multiple plants and individually analyzed for aflatoxin levels.

TABLE 15. Insect and Earthworm Population Observations on *Bt* Maize Hybrids and Isogenic Control Germplasm Plots During 1994 Field Trials[1]

Organism	Remarks
Black cutworm	All maize lines damaged equally
Corn root worm	Feeding damage and adult populations were observed to be equal; no discernible population differences associated with any maize lines
Flea beetles	All maize lines damaged equally
Leaf miners	All maize lines damaged equally
Ladybeetles	Beneficial insect, no discernible population differences associated with any maize lines
Pirate Beetles	Beneficial insect, no discernible population differences associated with any maize lines
Earthworms	Casting distribution was evaluated to be uniform (between 1.2 and 3 castings/square foot).

[1]Based upon visual observations by professional breeders and agronomists taken throughout the growing season. In addition, the following insects were inspected for but not seen in 1994: common stalk borer, wire worms, fall armyworm.

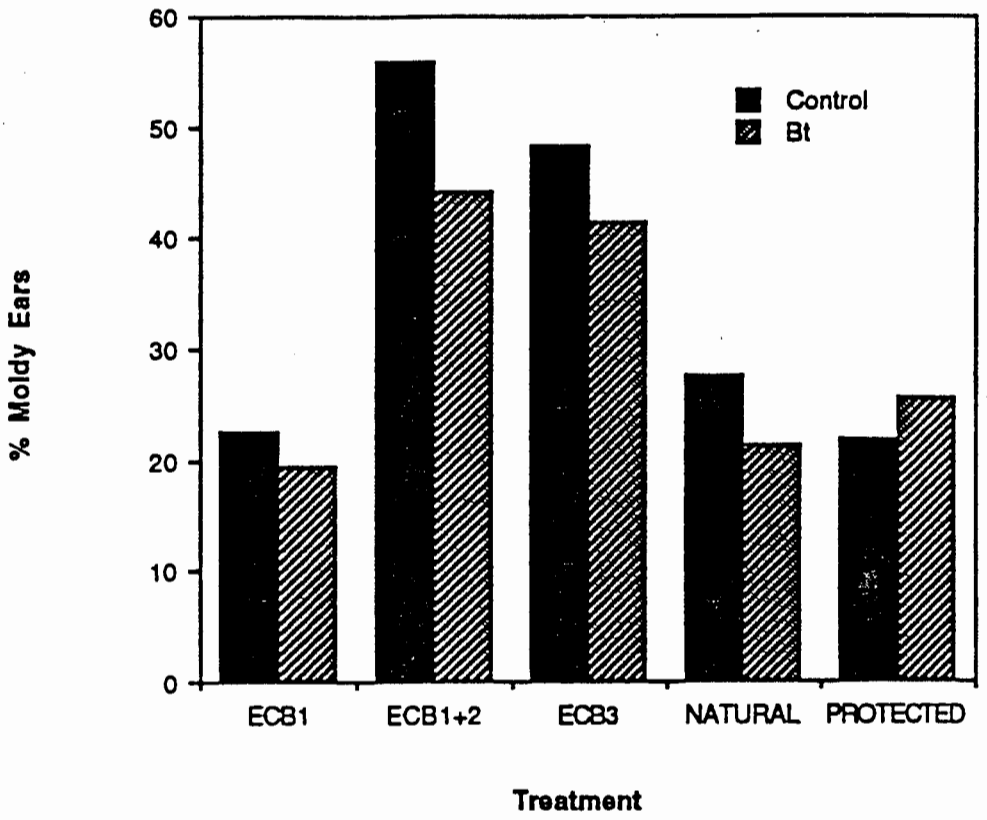

Figure 1. Incidence of fungal infestation (mold) present on ears harvested from a *Bt* maize hybrid and control hybrid grown under field conditions in 1993. All ears from six plots were visually inspected for presence of ear mold. Individual treatments as described in text.

APPENDIX 1

FIELD PERFORMANCE OF ELITE TRANSGENIC MAIZE PLANTS EXPRESSING AN INSECTICIDAL PROTEIN DERIVED FROM *Bacillus thuringiensis*

Bio/Technology 11: 194-200 (1993)

Field Performance of Elite Transgenic Maize Plants Expressing an Insecticidal Protein Derived from *Bacillus thuringiensis*

Michael G. Koziel*, Gary L. Beland†, Cindy Bowman, Nadine B. Carozzi, Rebecca Crenshaw, Lyle Crossland, John Dawson, Nalini Desai, Martha Hill, Sue Kadwell, Karen Launis, Kelly Lewis, Daryl Maddox, Kathryn McPherson, Moez R. Meghji†, Ellis Merlin, Richard Rhodes, Gregory W. Warren, Martha Wright and Stephen V. Evola

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Michael G. Koziel*, Gary L. Beland[1], Cindy Bowman, Nadine B. Carozzi, Rebecca Crenshaw, Lyle Crossland, John Dawson, Nalini Desai, Martha Hill, Sue Kadwell, Karen Launis, Kelly Lewis, Daryl Maddox, Kathryn McPherson, Moez R. Meghji[1], Ellis Merlin, Richard Rhodes, Gregory W. Warren, Martha Wright and Stephen V. Evola

CIBA-GEIGY Agricultural Biotechnology Research Unit, Research Triangle Park, NC 27709. [1]CIBA-GEIGY Seeds, 1301 West Washington, Bloomington, IL 61702. *Corresponding author.

We introduced a synthetic gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* into immature embryos of an elite line of maize using microprojectile bombardment. This gene was expressed using either the CaMV 35S promoter or a combination of two tissue specific promoters derived from maize. High levels of CryIA(b) protein were obtained using both promoter configurations. Hybrid maize plants resulting from crosses of transgenic elite inbred plants with commercial inbred lines were evaluated for resistance to European corn borer under field conditions. Plants expressing high levels of the insecticidal protein exhibited excellent resistance to repeated heavy infestations of this pest.

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European corn borer (ECB), *Ostrinia nubilalis* (Hubner), is a major pest of maize in North America and Europe. Yield loss of 3 to 7% per borer per plant can result from ECB feeding at various stages of plant growth[1]. Feeding results in physiological disruption of plant processes, leading to lower plant yield. Yield reduction from ECB in Illinois corn is estimated to exceed $50 million annually[2]. Chemical pesticides are effective against ECB, but inconvenience of scouting fields and determining treatment thresholds, narrow application windows on large corn plants, as well as the behavior of the insect, generally result in poor control. ECB typically has two generations annually; however, three or four generations can occur in a large area of its North American distribution[3]. First generation egg masses are laid on the underside of corn leaves beginning in May. Newly hatched larvae migrate into the whorl and feed on leaf material for 7 to 10 days. Third instar larvae then tunnel into the stalk where they feed, pupate, and emerge as second generation moths over an extended period during midsummer. These moths deposit their egg masses on the underside of leaves, most often in the region of the ear node. After hatching, most neonate larvae move to the leaf axils to feed on pollen accumulated at these sites and on sheath and collar tissue. Once ECB larvae begin feeding inside the collar, they are protected from control by chemical pesticides. Larvae begin to tunnel into the stalk after three to six weeks, most often in the ear region, where their feeding can result in severe yield loss from stalk breakage and/or from ears dropping to the ground.

ECB is susceptible to various insecticidal crystal proteins, or δ-endotoxins, produced by a number of strains of *Bacillus thuringiensis*, a gram positive, spore forming soil microbe (for review, see ref. 4). These crystal proteins are typically produced as large protoxins that are solubilized in the insect digestive tract, where they are proteolytically cleaved to produce an active insecticidal protein. The activated protein binds specifically to receptors in the midgut of the insect and brings about lysis of the cells by formation of pores[5,6]. Insecticidal proteins from *Bacillus thuringiensis* have been expressed in plants to confer insect tolerance[7-11]. Such expression has proven difficult when the native genes from *Bacillus thuringiensis* were used, necessitating the use of a truncated version of the native lepidopteran active genes for measurable protein and insecticidal activity in the transgenic plant. Use of a native δ-endotoxin coding region, which has a high A-T content, appears to lead to abnormally low gene expression in plants. Plants in general have a higher G-C content than that found in the δ-endotoxins, with maize having an even more pronounced preference for high G-C content in coding regions[12]. Modifying the coding sequence to increase the G-C content of the native gene results in a dramatic increase in expression of the insecticidal protein[13]. Our attempts to express detectable levels of CryIA(b) protein in maize using a truncated version of the native coding sequence have not been successful; therefore a modified coding sequence was used.

Transgenic plants expressing insecticidal proteins derived from *Bacillus thuringiensis* have been field tested and shown to resist insect feeding[14,15]. Maize transformation has not, until recently, been a routine procedure. Hence production of transgenic maize containing an insecticidal gene from *Bacillus thuringiensis* has lagged behind production of more readily transformed dicot plants. We report here the field performance of elite hybrid maize plants containing a synthetic gene encoding a truncated version of the CryIA(b) protein derived from *Bacillus thuringiensis* var. kurstaki HD-1[16]. Expression of the synthetic *cryIA(b)* gene in transgenic maize was effected using either the cauliflower mosaic virus (CaMV) 35S promoter or a combination of the phosphoenolpyruvate carboxylase (PEPC) promoter and a pollen specific promoter, both from maize. Our transgenic plants produced high levels of insecticidal protein and exhibited excellent protection against extremely high, repeated infestations with ECB.

Results

Introduction of a synthetic *cryIA(b)* gene into maize. A synthetic version of the *cryIA(b)* gene (construction to be described elsewhere) was utilized in this study after attempts to express the native gene in maize failed to produce detectable levels of CryIA(b) protein. As reported by others[13], increasing the G-C content of a *Bacillus thuringiensis* insecticidal protein gene greatly enhances its expression in plants. Modification of the native *cryIA(b)* coding region, which has a G-C content of about 38%, to possess a G-C content of about 65% produced a gene which is expressed at a high level in maize. This version of the *cryIA(b)* gene has about 65% homology at the nucleotide level with the native gene and is designed to resemble a maize gene in terms of codon usage. To our knowledge, this is the most radical alteration of a δ-endotoxin sequence to date. The gene used in this study encodes the first 648 amino acids of the 1155 amino acid protoxin and the complete sequence is available upon

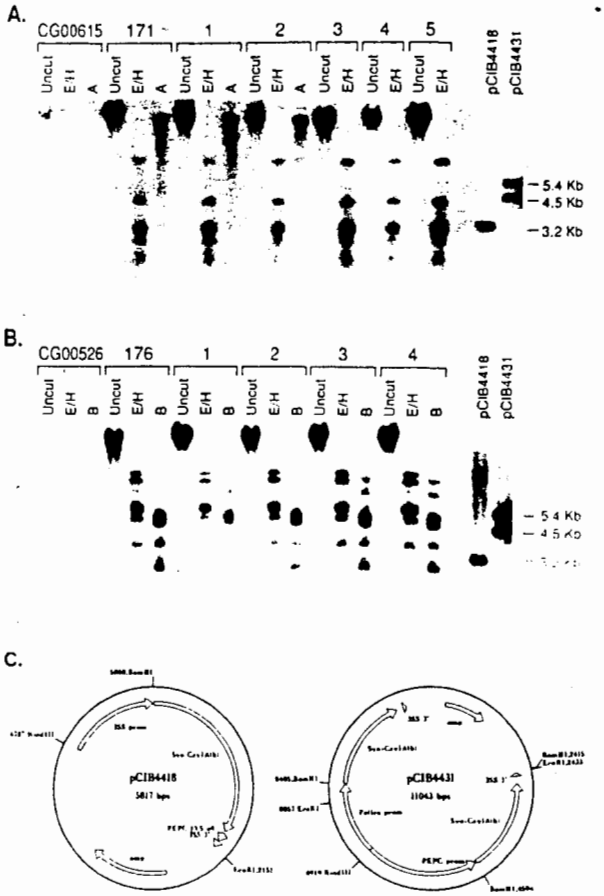

FIGURE 1. Southern blots of genomic DNA. The probe is specific for the synthetic *cryIA(b)* gene. (A) A parent plant and several progeny from event 171, the plants expressing the synthetic *cryIA(b)* gene using the CaMV 35S promoter. Lanes marked CG00615 contain DNA from a non-transgenic inbred with which the original transformants were crossed. Lanes marked 171: DNA from one of the original 171 regenerants. Lanes marked 1–5: DNA from five of the progeny from the 171 event. Lanes marked "uncut" are uncut DNA. Lanes marked E/H are cut with EcoRI and HindIII. Lanes marked "A" are cut with AflII. pCIB4418 is one of the plasmids used to transform the 171 event; it contains the 35S/syn-*cryIA(b)* gene. pCIB4431 is one of the plasmids used to transform the 176 event; it contains the PEPC/syn-*cryIA(b)* and pollen/syn-*cryIA(b)* genes. Both plasmids are cut with EcoRI and HindIII. (B) A parent plant and several progeny of the 176 event, the plants expressing the synthetic *cryIA(b)* gene using the PEPC and pollen promoters. Lanes marked CG00526 contain DNA from a non-transgenic inbred which was the recipient line for the transformation experiments. Lanes marked 176: DNA from one of the original 176 regenerants. Lanes marked 1–4: DNA from four of the progeny from the 176 event. Lanes marked "uncut" are uncut DNA. Lanes marked E/H are cut with EcoRI and HindIII. Lanes marked "B" are cut with BamHI. pCIB4418 and pCIB4431 are as described in (A) above. (C) Maps of the plasmids containing the synthetic *cryIA(b)* chimeric genes used to transform lines 171 (pCIB4418) and 176 (pCIB4431). The pollen/syn *cryIA(b)* and PEPC/syn *cryIA(b)* genes in pCIB4431 both contain the PEPC #9 intron in the 3′ untranslated region, as does the 35S/syn *cryIA(b)* gene.

request. The truncated protein encoded by this gene produces the same active insecticidal toxin as the full-length protoxin once it is proteolytically processed in the insect gut.

Chimeric *cryIA(b)* genes were introduced into a proprietary inbred line, CG00526, an elite cultivar of Lancaster parentage, using microprojectile bombardment of immature embryos, 14–15 days after pollination (see Experimental Protocol). Two independent events of transgenic maize expressing the synthetic *cryIA(b)* gene were chosen for further crossing and characterization. Initial transformants (multiple plants from each of the two events in this study) in the inbred line CG00526 were crossed to CG00526 as well as several other elite lines representing several heterotic groups. Event 171 contained the synthetic *cryIA(b)* gene under control of the CaMV 35S promoter and also contained a chimeric 35S/GUS gene for use as a scoreable marker. Event 176 contained a pair of tissue specific promoters controlling the expression of the synthetic *cryIA(b)* gene: the maize PEPC promoter[17], which is expressed in green tissues, and a maize pollen-specific promoter (to be described elsewhere). Both events contained a 35S/*bar* gene[18], used to confer resistance to phosphinothricin (PPT), as a selectable marker. The two events chosen for use in the field showed a 1:1 segregation ratio for phosphinothricin resistance, CryIA(b) expression, and ECB resistance when crossed with non-transformed plants. This observation, and other data (Southern analysis, Fig. 1), indicate a single site of insertion of the transgenes with a few copies of each gene. Parental and progeny plants from each event contain the same banding pattern in a Southern blot probed for the synthetic *cryIA(b)* gene. Uncut plasmids were used in the transformation experiments and therefore the banding pattern is more complex than that seen from cutting the plasmids themselves due to the random breakage of the plasmids prior to insertion into the plant genome.

Production of plants for field evaluation. Germination of immature embryos was employed to produce, in minimum time, the F1 hybrid plantlets for planting in the field. Immature embryos were germinated *in vitro* 14 to 15 days after pollination, allowed to develop, and transplanted to peat pots. When there was sufficient leaf material, samples were taken for analysis by β-glucuronidase (GUS) histochemical assay, PCR analysis for transgenes, growth in the presence of PPT, ELISA for CryIA(b) protein, and insect bioassay with ECB larvae. After analyses, plants were either discarded, shipped to Bloomington, Illinois, or retained in our greenhouse in North Carolina. Almost 1,000 plants were shipped to Illinois during June 1992, where the small peat pots containing the transgenic plants were transplanted into the field with 98% survival. Non-transgenic plants of inbred lines were planted in the same field over a six week period, starting in mid-May, to serve as controls and for pollinations.

Evaluation of transgenic maize plants in the field. When the plants reached approximately 40 cm in height, they were manually infested with neonate European corn borer larvae. About 300 larvae were applied to each plant per week for eight consecutive weeks for a total of 2,400 larvae per plant. The first four weeks of infestation correlated roughly with first generation timing and the second four weeks correlated with second generation infestation. ECB egg masses typically contain about 25 eggs. Thresholds of 0.5 egg mass per plant to as high as 4 egg masses per plant, depending on the relative tolerance of a particular maize hybrid, are generally used to determine if chemical control for second generation ECB is necessary. Assuming that every egg in an egg mass hatches, these 2,400 larvae would represent about 96 egg masses, half of which correspond to second generation infestation. Therefore our plants were challenged with 12 to 96 times the economic threshold of second generation ECB. The natural ECB pressure in surrounding fields was light and likely did not contribute significantly to the evaluation of these plants given the extremely high level of pressure from the repeated artificial infestations.

As indicated by the severe foliar and internal stalk damage

seen in the control plants, the ECB pressure we employed was more than adequate to evaluate the effectiveness of the synthetic *cryIA(b)* gene for ECB control. Table 1 and Figure 2 summarize the ECB damage ratings and their distribution for both first and second generation ECB infestations. All single cross transgenic families of both events were superior to the control inbred plants as assessed by either foliar feeding or internal stalk damage. With the exception of the CG00554 families, events 171 and 176 did not differ in performance for the above two traits. However, when averaged over all the families, event 176 derivatives had significantly better performance for foliar and internal stalk damage (data not shown). The average leaf damage rating for the best transgenic family, CG00554 X 176, was 1.6 compared to the non-transgenic inbred CG00554 which had a rating of 7.2.

Although first generation ECB control is important, the second generation can cause the greatest loss of yield, and is thus critical to the success of a commercial hybrid. The effectiveness of plants in controlling second generation larvae is ascertained by evaluating the extent of larval tunneling in the stalks. At the end of the field season, stalks from transgenic and control lines were split and examined for tunneling damage. The length of tunnels was significantly greater in control lines than in transgenic lines (Table 1 and Fig. 2). Transgenic plants expressing high levels of CryIA(b) protein had little or no tunneling damage. For example, the CG00554 X 176 family had an average of only 1.7 cm tunneling while the non-transgenic inbred had an average of 59 cm of tunneling damage. Typical differences in outer stalk damage and internal tunneling can be seen in Figure 3, as can the dramatic difference between transgenic and non-transgenic plants at the end of the growing season.

TABLE 1. Mean damage rating for first generation (ECB1) and second generation (ECB2), presented as foliar damage rating and tunnel length, respectively.

Maize Genotype	N	Mean ECB1 Foliar Damage Rating (1–9)[a]	Mean ECB2 Tunnel Length (cm)
CG00642 (Control)	10	6.3	40.7
CG00642 X 171	8	3.5	6.3
CG00642 X 176	13	3.0	2.7
CG00561 (Control)	10	7.3	60.8
CG00561 X 171	16	2.6	3.6
CG00561 X 176	7	2.1	2.3
CG00554 (Control)	10	7.2	59.3
CG00554 X 171	19	3.7	3.8
CG00554 X 176	13	1.6	1.7
CG00689 (Control)	5	7.1	28.3
CG00689 X 171	10	3.4	5.9
CG00689 X 176	8	2.8	2.4
CG00661 (Control)	5	6.7	113.8
CG00661 X 171	12	2.5	3.0
CG00661 X 176	14	2.9	3.9
CG00526 (Control)	19	6.2	41.3
CG00526 X 171	17	3.5	7.2
CG00526 X 176	15	3.5	5.2

N = number of plants characterized.

[a]Damage ratings were determined as follows:

1. No visible leaf injury.
2. Evidence of fine "window pane" damage only on the unfurled leaf where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
3. Evidence of fine "window pane" damage on two unfurled leaves where larvae plus corn cob grits fell into the whorl. No pin hole penetration of leaf.
4. Evidence of pin hole or shot hole feeding damage that penetrated the leaf on two or more leaves that emerged from the whorl (any lesion < 0.25" in length).
5. Elongated lesions and/or mid rib feeding evident on more than 3 leaves that emerged from the whorl. Lesion < 1" in length.
6. Several leaves with elongated lesions (0.75" to 1.5" in length) and/or no more than 1 leaf with broken mid rib.
7. Long lesions (> 1") common on about one-half of leaves and/or 2 or 3 leaves with broken mid ribs.
8. Long lesions (> 1") common on about two thirds of leaves and/or more than 3 leaves with broken mid ribs.
9. Most leaves with long lesions. Several leaves with broken mid ribs. Possibly stunted plants due to ECB1 feeding.

Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.

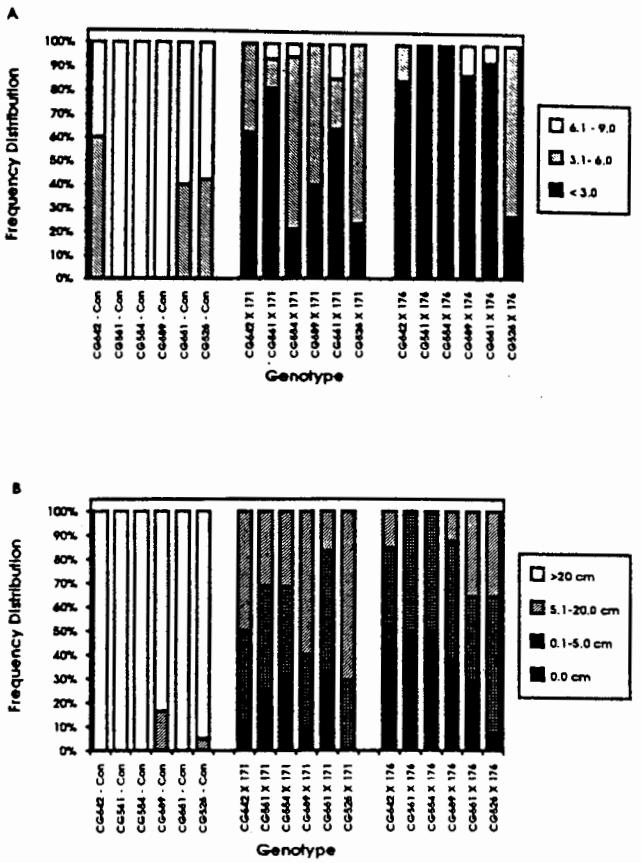

FIGURE 2. Frequency distribution of damage ratings for (A) first generation ECB (visual ratings 1–9) and (B) second generation ECB (tunnel length in cm). Event 171 = CaMV 35S/syn-*cryIA(b)* and event 176 = PEPC/syn-*cryIA(b)* and pollen/syn-*cryIA(b)*.

Laboratory assays were performed to determine the level of mortality brought about by ECB feeding on the transgenic plants. Leaf samples from the transgenic field plants were assayed for ECB activity by placing neonate larvae on leaf pieces in small petri dishes. Mortality was scored after 48 hours. All plants produced a high level of mortality within this time frame, with some at 100%. Typically, any larvae that were alive at 48 hours died by 72 or 96 hours. Results from some of these assays are shown in Table 2. We found a good correlation between the level of mortality at 48 hours and the level of CryIA(b) detected by ELISA (below). Plants with 100% mortality at 48 hours had the highest levels of CryIA(b) protein and also had the best overall performance ratings in the field.

Expression of chimeric *cryIA(b)* genes in maize. Transgenic plants with the best ECB damage ratings were analyzed for CryIA(b) protein levels using ELISA. Leaves from the field plants were sampled seven weeks post-transplant. Transgenic plants containing two synthetic *cryIA(b)* genes driven by the PEPC and pollen specific maize promoters produced over 1,000 ng CryIA(b)/mg soluble protein at week seven and were shown later in the season to exceed 4,000 ng

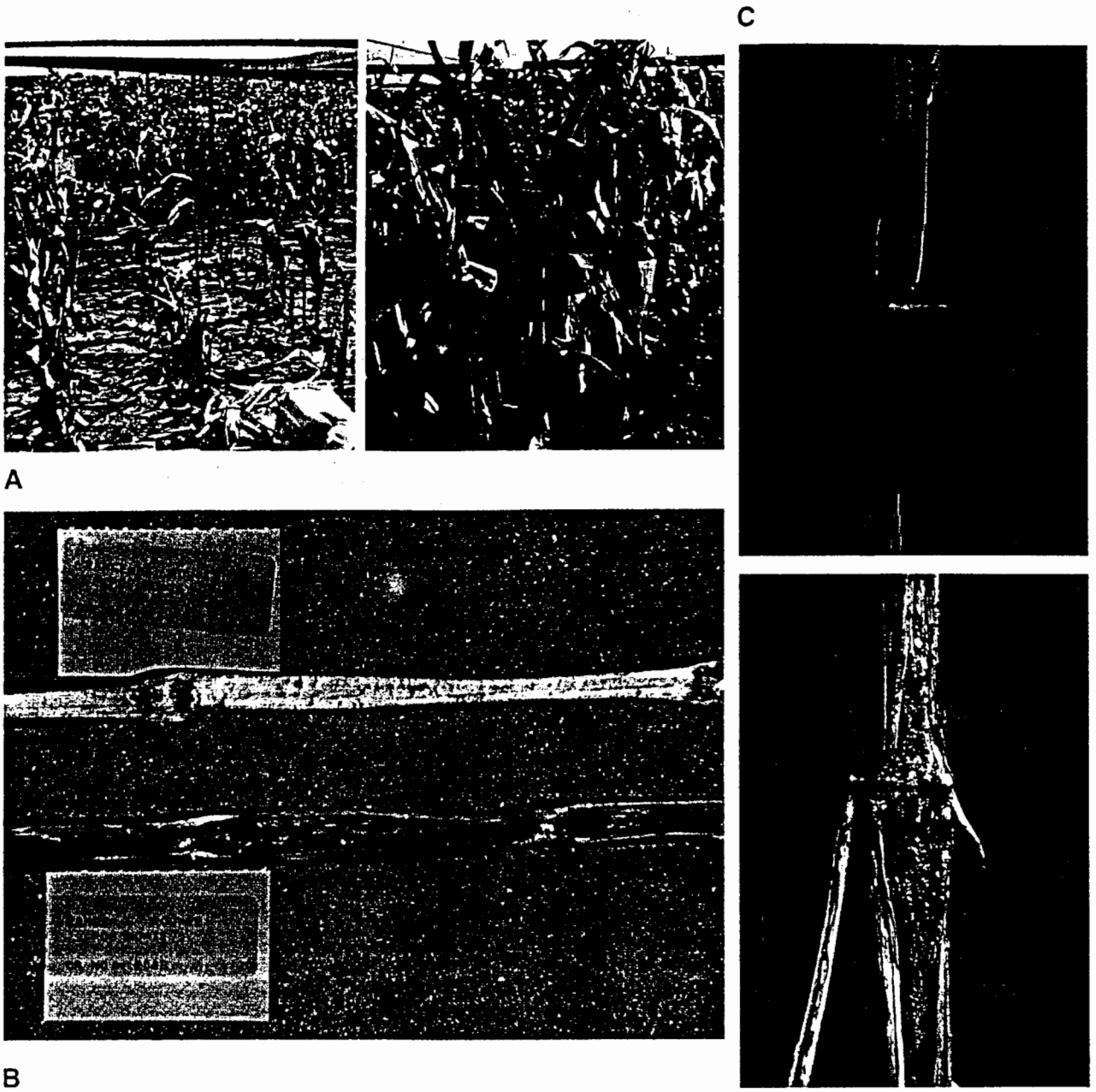

FIGURE 3. (A) View of non-transgenic (left) and transgenic (right) maize plants in the field at the end of the growing season. The ECB infestation pressure used to challenge these plants was high enough to cause complete loss of unprotected plants. (B) Split stalks from transgenic, upper, and non-transgenic, lower, plants showing the extent of tunneling damage caused by ECB in non-transgenic plants. (C) Outside view of stalks from transgenic, upper, and non-transgenic, lower, plants.

CryIA(b)/mg soluble protein. CryIA(b) protein levels in certain plants with the CaMV 35S promoter were as high at week seven as the levels in the PEPC and pollen promoter plants, but overall, these plants typically showed a much greater variation in CryIA(b) levels both within a particular cross with a given genotype and also among the genotypes. The levels of CryIA(b) in the PEPC and pollen promoter lines were all approximately equal in different genotypes. Table 3 presents typical ELISA values from five lines of each chimeric *cryIA(b)* construct.

Tissue specific expression patterns were evaluated by ELISA in several transgenic plants. Table 3 shows CryIA(b) protein concentrations in leaf, root, pith, pollen/anther, and kernels of selected plants. The CaMV 35S promoter is generally considered to be a constitutive promoter and as shown in Table 3, these plants had high levels of CryIA(b) in leaf, pith, and root. This correlates well with patterns of CryIA(b) expression observed in transgenic tobacco containing the same CaMV 35S promoter[11]. CryIA(b) was detected in the kernel but not in pollen/anther of the 35S plants. In the pollen and PEPC promoter plants, leaf expression of CryIA(b) was high while the pith and root expression levels were low. The green tissue surrounding the pith evidently contained sufficiently high levels of CryIA(b) to provide excellent insecticidal activity against ECB, as judged by the inability of ECB to penetrate the stalks significantly. While the level of CryIA(b) expressed in the pith of these plants was lower than that in the leaves, the level of expression was high enough to kill ECB, should any survive to penetrate the stalk. The high levels of CryIA(b) protein in pollen and low levels in kernel

produced by the combination of the PEPC and pollen promoters make this combination particularly attractive for producing a pattern of expression effective for controlling ECB while minimizing expression of CryIA(b) protein in seed and other tissues.

Discussion

Expression of a synthetic gene encoding a truncated version of the *cryIA(b)* insecticidal protein gene derived from *Bacillus thuringiensis* var. kurstaki HD-1 in elite single crosses of maize provides season long protection from repeated heavy infestations of European corn borer. Both first generation and second generation protection was obtained, even with an extremely heavy ECB infestation rate. Protection was obtained with plants hemizygous for the *cryIA(b)* gene; thus hybrid maize plants with only one transgenic parent can be protected. This is similar to the observation that heterozygous tobacco plants expressing a *cryIA(b)* gene were resistant to insects in a field situation[11,15]. Typical levels of CryIA(b) seen in plants containing the tissue specific chimeric genes were about 1,500 ng CryIA(b)/mg soluble protein, with levels as high as 4,000 ng/mg protein observed. The leaves in this test averaged about 8.5 mg total protein per gram fresh weight, so the plants were producing CryIA(b) protein at a "typical" level of 12,750 ng per gram fresh weight. The LC_{50} for ECB neonates is 20–30 ng/g diet. Adequate expression of CryIA(b) protein in pollen is important for control of second generation ECB larvae, for which pollen comprises a large portion of their diet during first and second instar. Transgenic plants containing the pollen and PEPC promoters expressing chimeric *cryIA(b)* genes produce the insecticidal protein in those parts of the plant consumed by both first and second generation ECB while minimizing expression in seed and other parts of the plants.

This is the first report of transformation of immature embryos from an elite maize inbred via microprojectile bombardment. Other reports of maize transformation have utilized suspension cultures[19,20] or callus cultures[21,22] of A188 X B73 crosses. Routinely, an average of one transformation event is recovered per 100 immature embryos bombarded, or one event for every 2.5 shots. The transgenic plants in this study were chosen from 23 events generated in 11 consecutive experiments. For the #171 event, 249 immature embryos were bombarded; 44 produced embryogenic callus on selection and the experiment yielded two transformation events. To obtain the #176 event, 251 embryos were bombarded, 37 of which produced embryogenic callus on selection. Although one embryo could in theory give rise to more than one transformation event, only one event is maintained from an embryo. Introduction of chimeric genes directly into elite inbreds could represent a significant savings in breeding time required to produce a commercial hybrid. As improved insecticidal genes become available, they can be introduced rapidly into commercial hybrids. The protection afforded by expression of the synthetic *cryIA(b)* gene in maize is significantly above that found thus far using traditional breeding methods. The ability to introduce improvements into elite inbreds, improvements which may not be within the genetic potential of the species, increases the alternatives available for management of pest problems and the rapidity with which they can become available.

The work presented here marks the first field evaluation of commercially relevant transgenic hybrid maize plants. One of the crosses made and evaluated employed the genotypes used in a commercial hybrid. Further, the plants tested represent two strategies for expression of foreign genes in plants for insect control. One strategy uses an essentially constitutive promoter, the CaMV 35S promoter, while the other uses conspecific tissue

TABLE 2. Percent mortality of ECB on leaf pieces from field grown transgenic maize.

Genotype	N[a]	Mean[b]	Range
35S Lines			
CG00642 X 171-4A	5	96	(85–100)
CG00554 X 171-4A	5	91	(75–100)
CG00526 X 171-4A	9	71	(70–100)
CG00661 X 171-4A	3	95	(90–100)
CG00554 X 171-4B	6	71	(55–90)
CG00689 X 171-13	7	75	(60–90)
CG00554 X 171-13	6	74	(55–100)
CG00561 X 171-14A	6	94	(85–100)
CG00689 X 171-14A	5	84	(70–90)
CG00554 X 171-14A	4	81	(70–95)
CG00716 X 171-14B	4	69	(50–80)
CG00615 X 171-15	2	90	(90)
CG00554 X 171-15	3	75	(55–95)
CG00526 X 171-15	1	65	(65)
CG00716 X 171-16AB	2	90	(80–100)
CG00689 X 171-16AB	2	68	(65–70)
CG00526 X 171-16AB	2	78	(70–85)
CG00661 X 171-16AB	5	99	(95–100)
CG00689 X 171-18	6	75	(65–90)
CG00554 X 171-18	3	72	(50–85)
CG00526 X 171-18	1	95	(95)
PEPC/Pollen Lines			
CG00689 X 176-11	5	96	(90–100)
CG00554 X 176-11	4	96	(85–100)
CG00526 X 176-11	2	100	(100)
CG00661 X 176-11	8	99	(95–100)
CG00642 X 176-10	2	100	(100)

[a]N = Number of plants assayed using 20 neonate larvae per plant
[b]Mean mortality was scored at 48 hours. Control plants produce an ECB mortality of 0-20%. Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.

TABLE 3. Quantification of CryIA(b) protein levels in various tissues of maize.

Genotype	Leaf	Root	Pith	Pollen/ Anther	Kernel
35S Lines					
CG00554 X 171-18	513 ± 244 (n=4)	596	288	NT	53
CG00554 X 171-4A	1732 ± 39 (n=2)	NT	NT	NT	NT
CG00554 X 171-13	767 ± 842 (n=8)	1209	4381	0	274
CG00554 X 171-14A	655 ± 554 (n=7)	3348	2440	0	149
CG00615 X 171-16BB	283 ± 227 (n=7)	74	71	0	NT
PEPC/Pollen Lines					
CG00661 X 176-10	1703 ± 378 (n=9)	52	60	260	15
CG00554 X 176-11	1288 ± 583 (n=12)	54	113	418	16
CG00642 X 176-10	1138 ± 188 (n=4)	NT	NT	NT	NT
CG00689 X 176-11	1077 ± 108 (n=3)	NT	NT	340	NT
176-11 X CG00526	1842 ± 345 (n=2)	47	53	NT	18

Values are ng CryIA(b)/mg soluble protein ± standard deviation.
Values for control plants analyzed by ELISA are 0 ng.
Plants from event 171 contain the chimeric CaMV 35S/*cryIA(b)* gene while plants from event 176 contain the chimeric PEPC/*cryIA(b)* and pollen-specific/*cryIA(b)* genes.
NT = not tested

specific promoters. Both types of plant produce high levels of CryIA(b) insecticidal protein. The plants containing chimeric insecticidal genes expressed using tissue specific promoters produce a consistently higher level of the CryIA(b) protein, but one must be mindful that these are only single transformation events leading to each of the different lines. Further events are under analysis to see how broadly these findings can be applied. Nonetheless, it is clear that expression of a high level of insecticidal proteins derived from *Bacillus thuringiensis* can protect maize plants from insect attack in a field environment.

Transgenic plants expressing insecticidal genes present a new tool for use in an integrated pest management strategy. Transgenic plants deliver the δ-endotoxins in a manner quite different from that of *Bacillus thuringiensis* microbial sprays. The larvae are exposed to the insecticidal protein in a more uniform manner on a constant basis and from the earliest possible time. The neonate larvae are the most susceptible stage and are exposed as soon as they begin to feed on the plant. They do not have an opportunity to avoid ingesting the insecticidal protein. As with any other insect control mechanism, development of resistance to the expressed insecticidal proteins might be a possibility. There are various strategies for preventing, or at least delaying, the onset of such resistance. As is true for any other insect control method, use of transgenic plants should represent only part of the total insect control practice. As agricultural biotechnology continues to mature, new strategies for insect control will become available. These will undoubtedly entail different mechanisms for insect control than are presented by the *Bacillus thuringiensis* δ-endotoxins. Use of a variety of such genes, either in a linear progression, on a revolving basis, or in various combinations, should combat the development of resistance to any single mechanism or gene product.

Experimental Protocol

Transformation vectors. Vectors used to transform maize are all derivatives of pUC18 or pUC19[23]. pCIB4418 contains a synthetic gene encoding the amino terminal 648 amino acids of CryIA(b) from *Bacillus thuringiensis* var. kurstaki HD-1[16], fused with the CaMV 35S promoter[24]. The sequence of this synthetic gene is available upon request from the corresponding author. pCIB4431 contains two chimeric *cryIA(b)* genes. The first is under control of the maize PEPC promoter[17] and the second is under control of a pollen specific promoter derived from maize (cloning and characterization to be described elsewhere). pCIB3064 contains a plant expressible *bar* gene[18] driven by the CaMV 35S promoter to provide resistance to phosphinothricin. pCIB3007 contains a GUS gene under control of the CaMV 35S promoter with a 144 NT leader derived from the CaMV 35S transcript (A. Montoya, unpublished data). A copy of the maize Adh1 intron #1 has been inserted into the 5′ leader of this gene.

Transformation and embryo rescue of progeny. Immature embryos (1.5 to 2.5 mm in length) of Ciba maize inbred CG00526, developed from a Lancaster-type population, were aseptically excised 14–15 days after pollination from surface-sterilized, greenhouse-grown ears and plated scutellum up on callus initiation medium, 2DG4 + 5 mg/l chloramben. 2DG4 medium is Duncan's "D" medium[25] modified to contain 20 mg/l glucose. Plasmid DNA was precipitated onto 1 μm gold microcarrier as described in the DuPont Biolistic manual. For event 176, 6.15 μg of pCIB4431 and 1.80 μg of pCIB3064 were used per 50 μl of microcarrier. For event 171, 2.72 μg of pCIB4418, 1.8 μg of pCIB3064, and 2.72 μg of pCIB3007 were used per 50 μl of microcarrier. Thirty-six embryos per plate were bombarded using the PDS-1000He Biolistic device. Tissue was placed on the shelf 8 cm below the stopping screen shelf and a 10 × 10 μm stainless steel screen was used with rupture discs of 1550 psi value. After bombardment, embryos were cultured in the dark at 25°C for one day, then transferred to callus initiation medium containing 3 mg/l PPT and incubated in the dark at 25°C. Resultant embryogenic tissue was transferred to callus maintenance medium, 2DG4 + 0.5 mg/l (2,4-dichlorophenoxy)acetic acid (2,4-D) containing 3 mg/l PPT and subcultured every 2 weeks. Twelve weeks later, tissue was cultured at 25°C on a modified Murashige and Skoog medium (MS)[26] containing 3% sucrose, 0.25 mg/l 2,4-D and 5 mg/l benzylaminopurine with 16 hours of light (50 μE/m-2/s-1) per day to initiate regeneration. Two weeks later the tissue was transferred to MS medium containing 3% sucrose. After 4 to 10 weeks, regenerated plants were cultured on MS medium modified to contain half the concentration of salts and 3% sucrose. Transformed plants were identified using the chlorophenol red (CR) assay to test for resistance to PPT[27], the histochemical GUS assay where appropriate[28] and PCR for sequences in the 35S promoter and the synthetic *cryIA(b)* gene. Positive plants were moved to the greenhouse for additional testing and crossing with various inbreds. Event 171 produced a total of 33 transgenic plants and event 176 produced a total of 38 transgenic plants. To minimize the time required to obtain T1 plants for field testing, we germinated immature embryos. Fourteen to sixteen days after pollination, the ear tip (25–50 kernels) was removed. The excised ear piece was surface-sterilized and individual embryos removed and plated on B5 medium[29] containing 2% sucrose. The method is described in detail by Weymann et al.[30].

Insect bioassays. When plants in the field reached about 40 cm of extended leaf height, infestation with laboratory-reared ECB larvae was begun on both the transgenic plants and non-transgenic controls. About 300 neonate larvae mixed with corn cob grits were introduced into the whorl of each plant using a Davis inoculator[31]. Infestations continued on a weekly basis for four weeks to simulate first generation corn borer (ECB1). Starting two weeks after the initial infestation, each plant was rated weekly for four weeks using a modification of the 1 to 9 scale described by Guthrie[32]; we reserved a rating of 1 for plants with no damage whatsoever. See legend to Table 1 for details. A mean ECB1 damage rating score was calculated for each transgenic plant and non-transgenic control plant. As each plant reached anthesis, 300 larvae/plant were applied weekly for four weeks to simulate second generation infestation (ECB2). One hundred neonate larvae in corn cob grits were introduced into the leaf axil at the primary ear and at the leaf axil one node above and below the primary ear node. About 50 days after the initial ECB2 simulated infestation, selected stalks from each of the two events in all lines as well as non-transgenic control plants were harvested. The extent of internal ECB tunneling damage in a 92 cm section of stalk 46 cm above and below the primary ear node was measured on a subset of transgenic and control plants. European corn borer assays using pieces of leaf from field plants were carried out in the laboratory. One to four 4 cm sections were cut from an extended leaf and placed on a moistened filter disc in a 50 X 9 mm petri dish. Five neonate European corn borer larvae were placed on each leaf piece and the petri dishes were incubated at 29.5°C. Leaf feeding damage and larval mortality data were scored after 48 hours.

CryIA(b) protein quantification. Detection and quantitative determination of the amount of CryIA(b) protein expressed in transgenic plants was monitored using enzyme-linked immunosorbant assays (ELISA)[33]. Immunoaffinity purified polyclonal rabbit and goat antibodies specific for the insecticidal crystal proteins from *Bacillus thuringiensis* subsp. kurstaki HD-1 were used to determine ng CryIA(b) per mg soluble protein from crude extracts of leaf samples. The sensitivity of the double sandwich ELISA is 1–5 ng CryIA(b) per mg soluble protein using 50 μg of total protein per ELISA microtiter dish well. Corn tissue extracts were prepared by grinding leaf tissue in gauze lined plastic bags using a hand held ball-bearing homogenizer (AGDIA, Elkart, IN.) in the presence of extraction buffer (50 mM Na_2CO_3 pH 9.5, 100 mM NaCl, 0.05% Triton, 0.05% Tween, 1 mM phenylmethylsulfonyl fluoride and 1 μM leupeptin). Protein determination was performed using the Bio-Rad (Richmond, CA) protein assay.

Statistical analysis. Analyses of variance were performed to compare the two events and to compare them with the control plants for the average foliar feeding damage ratings and for cm internal tunneling damage in the 92 cm stalk section using the general linear models (GLM) procedure in SAS[34] with the associated least significant differences (LSD) function. Similarly, the events and controls were compared to each other within all families.

Southern blot analysis. Genomic DNA was isolated from maize plants and processed for Southern blot analysis using standard procedures[35]. A gel purified fragment containing only the synthetic *cryIA(b)* gene was used to generate a random-primed ^{32}P probe. Southern blots were prepared and hybridized using standard procedures and washed at 65°C in 0.3 X SSC.

Acknowledgments

We thank Mary-Dell Chilton for assistance in the preparation of this manuscript; Ben Miflin and Dean Christensen for critical comments on the manuscript; Rich Lotstein for securing the required field test permits; Joyce Craig for technical assistance, and Fasica Woldeyes and Wayne Anderson for help in care and transport of the plants.

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APPENDIX 2

YIELD LOSS DUE TO EUROPEAN CORN BORER IN NORMAL AND TRANSGENIC HYBRIDS

48th Annual Corn & Sorghum Research Conference
1993

We are looking at opportunities for moving identity-preserved grains to Mexico. We conducted a major study that demonstrated the future of IP corn in the North American market.

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Yield Loss Due to European Corn Bore' in Normal and Transgenic Hybrids

Dean Christensen, Gary Beland and Moez Meghji
Ciba Seeds
P.O. Box 18300
Greensboro, North Carolina

Our success as members of the hybrid seed corn industry is depe lent on developing products which will help our farmer customers to be su essful. New hybrid varieties must be high yielding, but they must also b reliable from year to year in order to help farmers overcome the risks w ich are inherent in agricultural production. One of those risks is yield lo due to feeding damage by the European corn borer (ECB), *Ostrinia ubilalis* (Hubner). The cost of this damage has been estimated to exceed $5(million per year in Illinois alone (Briggs and Guse, 1986). Chemical con ols are available, but they are not widely used due to inconvenience of couting fields and determining treatment thresholds, narrow application indows, and short residual effectiveness. Genetic resistance of corn to ECB as been the subject of much research, but progress in elite germplasm, espe ally for second generation resistance, has been difficult. European corn bore damage is still a major problem for corn growers.

In 1990, Ciba Seeds began a series of tests to assess yield loss caused by ECB damage to elite hybrids. In 1992, Ciba Seeds conducted field tests of transgenic corn plants expressing an insect control protein derived from *Bacillus thuringiensis* (Bt). Controlled yield studies were conducted in 1993 which contained ECB infestation treatments and compared a Bt-expressing hybrid to isogenic non-Bt controls. This paper will summarize the results of these studies with the purpose of describing the risk of yield loss due to ECB and the opportunity to reduce that risk with ECB-resistant transgenic hybrids.

DEAN CHRISTENSI

YIELD LOSS STUDIES WITH NORMAL HYBRIDS

Numerous studies have characterized the effect of ECB feeding on corn yield. Jarvis et. al. (1991) described the results of 2nd-generation infestation on hybrids which varied in resistance to ECB. Yield losses were greatest in susceptible hybrids, but even hybrids in which both parents were characterized as resistant averaged a 6.6% yield loss at an infestation rate of only 1/2 egg mass per plant. Bode and Calvin (1990) showed that yield loss ranged from 2.4% to 5.9% per ECB larva per plant, depending on the time of infestation. Similar estimates of 3.0%–6.6% yield loss per ECB larva per plant have been recommended for use in making insect management decisions (Showers et. al., 1992).

The potential for yield loss due to ECB can vary widely across environments. Showers et. al. (1992) described numerous biological, and climatic factors which can affect ECB survival and corn infestation rates. The interaction of these factors has resulted in large year to year variation in ECB infestation rates. Briggs and Guse (1986) summarized results for forty years of fall European corn borer surveys in Illinois. An average of 1.19 larvae per stalk were found over the whole time period, but means for individual years ranged from 0.26 to 4.20. Even after infestation occurs, the amount of yield loss caused by ECB can be affected by the physiological condition of the plants. Both Lynch (1980) and Godfrey et. al. (1991) presented data showing that yield loss per unit infestation can be twice as large in drought conditions when compared to more favorable conditions.

Ciba Seeds conducted a series of studies during the past four years to measure yield loss due to second generation ECB. Infestation treatments were modified in some trials as we gained experience, and genetic entries were updated each year. Therefore, a combined analysis of all trials is not possible. For the data presented here, trials contained elite commercial and precommercial hybrids. A standard split plot design was used, with infestation treatments as main plots and genotypes as sub-plots. In 1990 the experimental unit was a two-row plot 18 feet long. In subsequent years, the experimental unit was a four-row plot, and the center two rows of the plot were used for treatments and data collection. Main plots consisted of two treatments, infested and protected. Three replicates of the treatments were used in all trials. The second generation infestation treatment consisted of two infestations of 100–150 ECB larvae applied at and immediately following pollen shed. The protected treatment was sprayed as needed with commercially available insecticides to suppress natural ECB infestations. At Bloomington, Illinois, a trial of similar design consisted of three main plot treatments, infested, protected and a natural treatment which did not receive applications of ECB larvae or insecticide. Experiments were combine-harvested to determine yield.

Figure 1 shows yield results of infested and protected treatments, averaged over hybrids, for four representative trial-year combinations. Treatment differ-

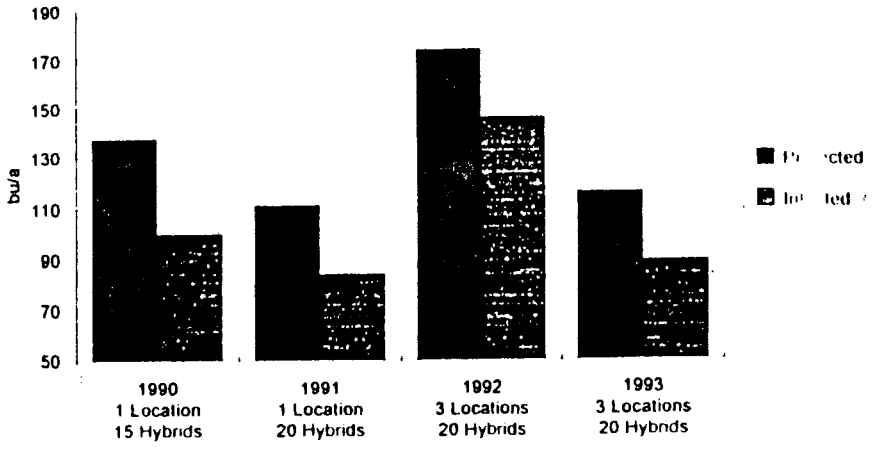

Figure 1 Average yields of elite hybrids within treatments of insecticide protect[ed] and applied infestation of second generation ECB larvae.

ences were significant at the 0.05 probability level for each trial. In th[ree] of these four trials average yields of infested plots were reduced by mor[e] than 20% relative to the control. Individual hybrids within a trial typicall[y] had second generation ECB damage that resulted in yield losses of 15% to [a]bove 30%. Other researchers have reported similar high levels of yield l[o]ss in heavily infested plots (Jarvis et. al., 1991; Klenke, et. al., 1986).

The high level of artificial infestation used in these trials could be c[on]sidered to have caused damage similar to the worst possible natural infest[a]tions. The uninfested, unsprayed treatment included in experiments at Bloomi[ng]ton, Illinois allowed a measure of yield loss due to natural ECB infesta[t]ions. Figure 2 presents yield results from four years of these trials for prot[ec]ted, unprotected and artificially infested treatments. Differences between all [tr]eatments within each trial were significant at the 0.05 level of probabil[i]ty in 1990 and 1993. In those years yield losses due to natural ECB were [6].1% and 8.7%, respectively, relative to the protected treatment. In 1991 a s[e]vere natural ECB infestation caused yield losses essentially equal to our high artificial infestation treatment. In contrast, European corn borer popul[a]tions were greatly reduced in 1992, resulting in no yield loss due to natural in[fes]tation.

Two conclusions can be drawn from these studies and from previ[ously] published research. First, heavy second generation ECB infestations can [ca]use severe yield losses, typically above 20%, in elite hybrids. Second, a[c]tual yield losses in commercial fields are highly variable from year to ye[ar] and can approach the maximum seen in artificially infested plots. The ex[tre]me differences for amount of yield loss from natural infestations at Bloomi[ng]ton

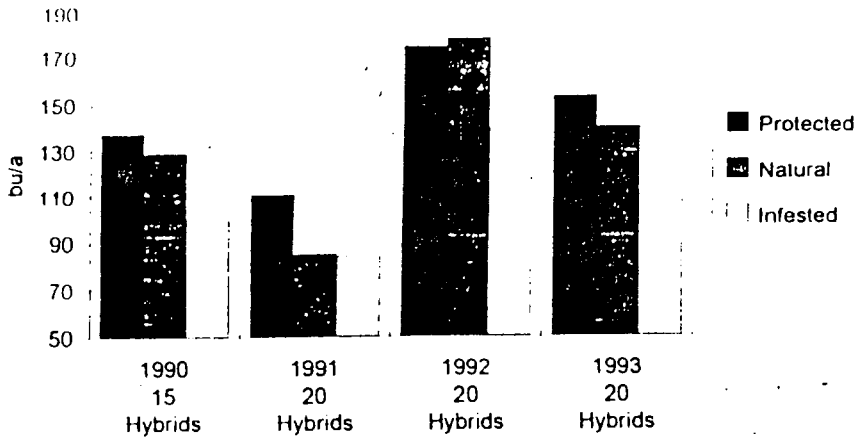

Figure 2 Average hybrid yields of elite hybrids at Bloomington, Illinois within treatments of insecticide protection, natural ECB infestation and applied infestation of second generation larvae.

in 1991 and 1992 may be relatively rare. However, they are a reminder of the risk and uncertainty which European corn borer poses to farmers.

YIELD LOSS STUDIES WITH TRANSGENIC INSECT RESISTANT CORN

Materials and Methods

The development and initial field tests of Ciba Seeds' transgenic corn was described by Koziel et. al. (1993). A synthetic gene encoding a version of the Cry1A(b) protein derived from *Bacillus thuringiensis* was introduced into Ciba Seeds elite inbred line 00526. European corn borer is known to be susceptible to the Cry1A(b) protein. The transformed plants included in the initial studies, particularly those derived from transgenic event 176, showed extremely high levels of resistance to both generations of European corn borer. Based on these promising results, trials were planned for 1993 to test performance of hybrids containing a transgenic parent. Inbred 00526 plants from event 176 were self-pollinated and progeny tested to identify homozygous isolines which either contained the Bt gene (Bt+) or did not contain the Bt gene (Bt−). These isoline selections were then crossed to an elite tester inbred to produce hybrid seed for field tests. In addition, standard non-transformed plants of inbred 00526, designated wild type (WT), were crossed to the same tester to produce a hybrid which did not contain the Bt gene and was not derived from transformed plants.

The three versions of the hybrid (Bt+, Bt− and WT) were t ted for performance under corn borer pressure, utilizing a split-block type exp riment with infestation treatments as main plots and hybrids as sub-plots. Th experi ment contained three replicates of each treatment. Four-row pl ts were planted. Only the center two rows of each plot were used for treatm nts and data collection in order to reduce possible confounding effects due t migra tion of ECB larvae. All four rows of the experimental plots were det eled in order to include the trials with other corn plantings and still satisfy r ulatory requirements for isolation of transgenic plants. Adequate pollen for e: forma- tion in the experimental plots was provided by planting non-detasselec borders of normal hybrids on each side of every experimental plot. Five w le-plot treatments were used: 1) ECB1: Two applications of 100–150 EC larvae at the mid-whorl stage of growth to simulate heavy first generati n ECB infestation, followed by insecticide protection as needed for second g eration infestations, 2) ECB1+2: Two applications of 100–150 ECB larv at the mid-whorl stage of growth followed by two applications of 100–1 larvae at mid-pollen shed to simulate heavy first and second generation int station. 3) ECB2: Insecticide treatment as needed for natural first generati ECB, followed by two applications of 100–150 ECB larvae at mid-polle shed to simulate heavy second generation infestation, 4) NATURAL: No app cations of ECB or insecticide in order to measure the effects of natural ECB nfesta- tions, 5) PROTECTED: Insecticide applied as needed throughout th season in order to provide an uninfested control for all genotypes. A com mercial synthetic pyrethroid insecticide was used for all insect protection appl ations.

The experiment was planted at six locations in the 1993 season: eward, Nebraska; Winterset, Iowa; Marion, Iowa; Shelbyville, Illin and Bloomington, Illinois (Center Farm and North Point). The Winters , Iowa location was damaged by excess water, and data from that location as not used in the combined analysis. Because of limited seed supply, pl s were thinned to about 19,000 plants per acre. Plots were hand harvested order to insure collection and isolation of transgenic grain. Yield from ea on the ground was recorded separately from the yield of ears on upright alks in order to allow calculation of combine harvestable yield and total yi l. Data were collected at all locations for final population, ECB1 damage, lat season staygreen, plants broken above the ear, plants broken below the ear. opped ears, yield and grain moisture at harvest. The rating scale desc ed by Guthrie, et. al. (1960) was used for evaluating ECB1 leaf feeding d mage.

Analyses of variance were obtained for all traits using data fr m five locations. All effects except replicates and locations were consider fixed. The significance level P=0.05 was used for all data comparisons. The mma- ries contained in this paper focus on harvestable yield results and ddress three main issues: 1) Relative performance of the Bt+ hybrid wi in the treatments which received artificial or natural ECB infestations, 2) erfor- mance of the Bt+ hybrid in infested treatments relative to its perf mance

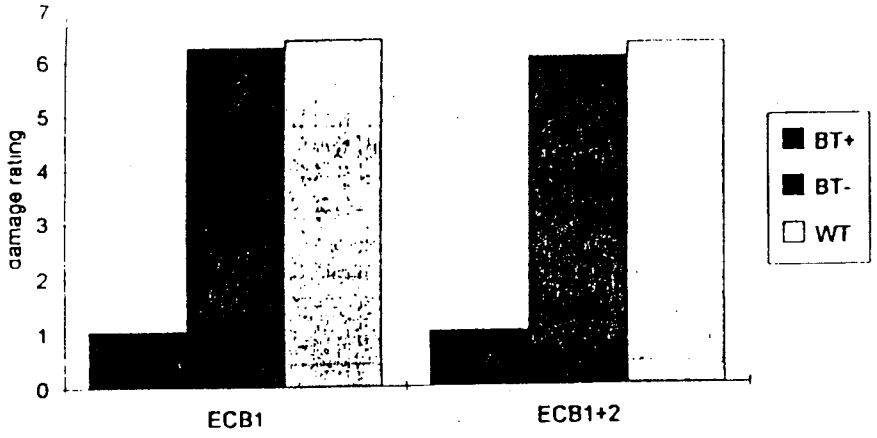

Figure 3 Average first generation ECB damage ratings for Bt+, Bt− and WT hybrids in the ECB1 and ECB1+2 treatments.

in the protected treatment, and 3) Relative performance of the Bt+ hybrid within the protected treatment.

Hybrid Performance within Infested Treatments

Figure 3 shows hybrid ratings from the ECB1 and ECB1+2 treatments for first generation leaf feeding. The Bt+ hybrid had no feeding damage, whereas both the Bt− and WT hybrids showed extensive damage. However, these differences were not reflected in the yield results. Figure 4 summarizes

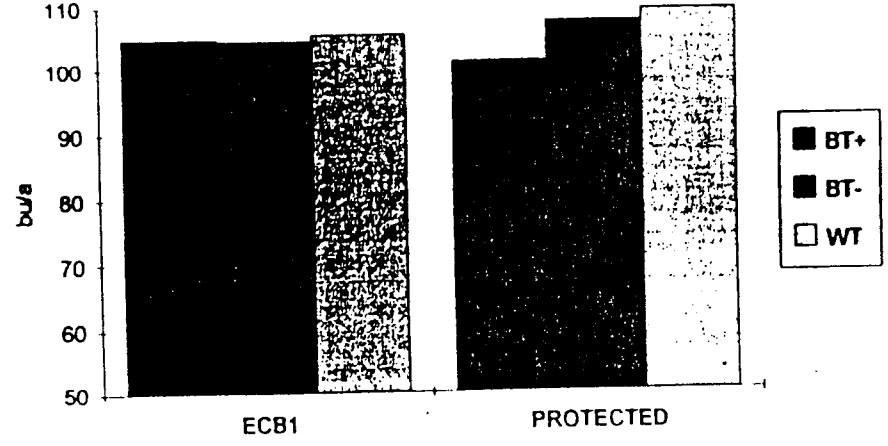

Figure 4 Average yields for Bt+, Bt− and WT hybrids in the ECB1 and PROTECTED treatments.

hybrid yield within the ECB1 and PROTECTED treatments. There were no differences in performance of the three genotypes within the ECB1 treatment, and there was no difference between treatment means for ECB1 and PROTECTED. For this set of 1993 locations there was no adverse yield effect of first generation infestations. Previous studies have shown sizable yield losses due to first generation ECB (Bode and Calvin, 1990; Lynch 1980). Heavy rainfall throughout the corn belt during the early summer of 1993 may have reduced the numbers of first generation ECB which survived to bore into the corn stalk. However, the exact reasons for these results are unclear.

Individual genotypes had the same level of performance across the ECB1+2 and ECB2 treatments (Figure 5), which once again indicated no effect of first generation infestations. The Bt+ hybrid demonstrated a significant yield advantage in both of these treatments, indicating a high level of resistance to second generation ECB. Yield of the Bt+ hybrid was first over 25 bushels per acre, or 35%, greater than the susceptible genotype. There were also large differences between the genotypes in these treatments for other agronomic traits. Susceptible hybrids senesced sooner than the Bt+ hybrid and therefore had significantly less late season staygreen and lower grain moistures at harvest. Differences in stalk quality were apparent. The Bt+ hybrid had significantly fewer broken stalks above the ear. Differences among hybrids for stalks broken below the ear were not significant for all comparisons, but means for the Bt+ hybrid were consistently lower than the susceptible hybrids.

Differences among hybrids within the NATURAL treatment were not significant for the total set of locations. However, at two of the five sites,

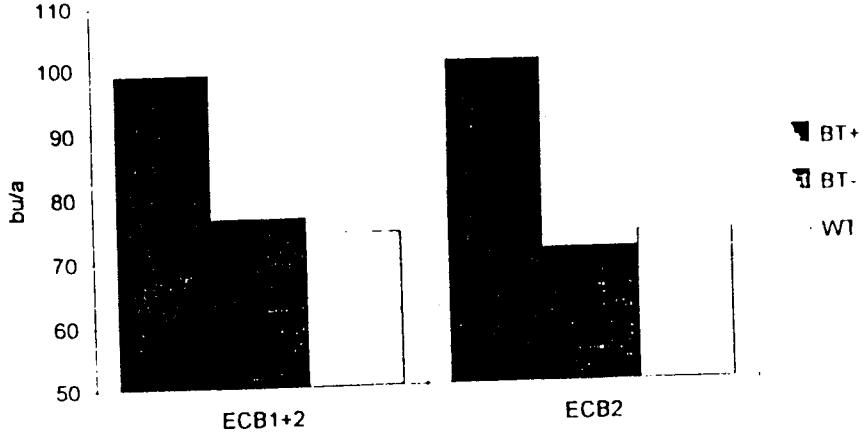

Figure 5 Average yields for Bt+, Bt− and WT hybrids in the ECB1+2 and ECB2 treatments

hybrids which were susceptible to ECB showed a high level of physical damage from natural infestations. At these two sites the Bt+ hybrid yield was noticeably greater than the yield of the susceptible genotypes (Figure 6). Although these within-location differences among the hybrids were not significant (P=0.05), the results were consistent with the larger yield differences observed in artificially infested plots.

Bt+ Hybrid Performance Across Treatments

There were no yield differences for the Bt+ hybrid between the various infestation treatments and the PROTECTED treatment (Figure 7). The artificially infested treatments for second generation ECB were a severe test of the level of insect control in the Bt+ hybrid. High numbers of ECB larvae were applied for the ECB1+2 and ECB2 treatments, but only limited damage was observed. The Bt+ hybrid maintained a consistent yield level across each of the infestation treatments of this study.

Hybrid Performance within the Protected Treatment

The transformation process for corn is currently a random event. There is a possibility that new genes may be inserted at a site in the genome which disrupts the activity of native genes. There is also a possibility the expression of the transgene will require sufficient resources to reduce the overall fitness, or performance, of the plant. For these reasons hybrids in the protected treatment were closely observed throughout the 1993 growing season. No

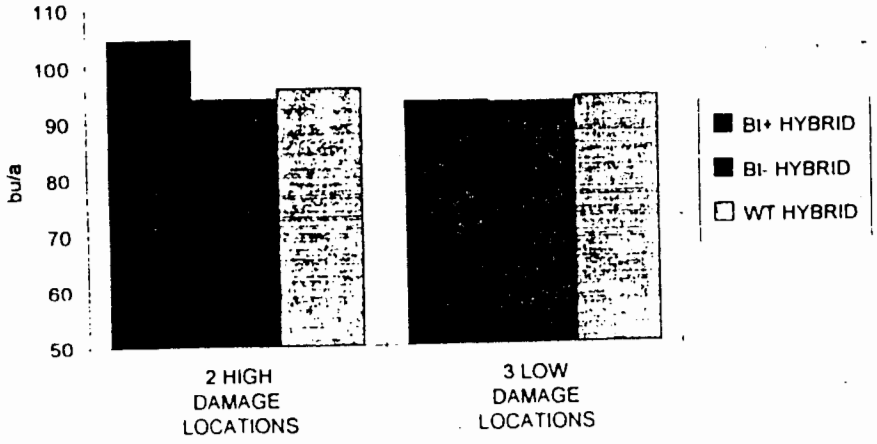

Figure 6 Average hybrid yields for Bt+, Bt− and WT hybrids from the NATURAL treatment at two sets of locations. Test sites were grouped by level of physical damage caused by ECB feeding.

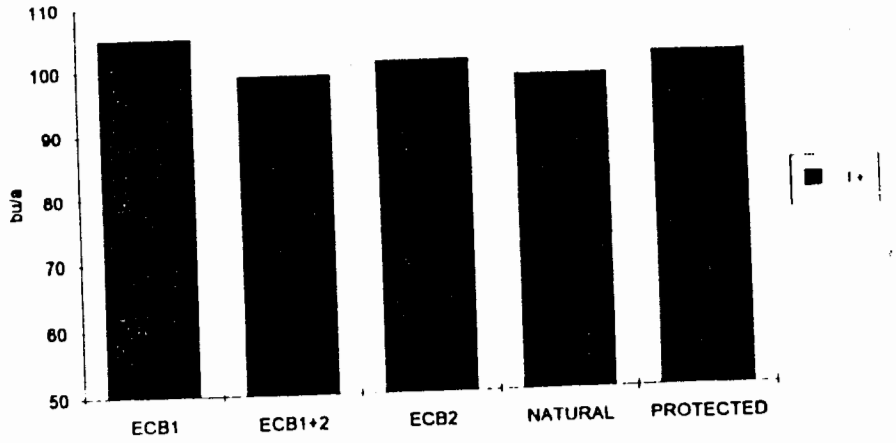

Figure 7 Average yield of Bt+ hybrid in ECB1, ECB1+2, ECB2, NATURAL and PROTECTED treatments.

morphological or developmental differences were noted among the three hybrids. All hybrids flowered during the same period and were equal for height and other morphological characteristics. Figure 4, already discussed above, compares hybrid yields in the ECB1 and PROTECTED treatments. Since there was no yield effect of the first generation ECB infestation in this study, the ECB1 treatment can also be considered as an assessment of Bt+ hybrid performance in the absence of insect pressure. There were no significant differences for yield among hybrids in either the ECB1 or PROTECTED treatments. The slightly lower yield of the Bt+ hybrid in the PROTECTED treatment appeared to have been related to significantly reduced plant populations in the Bt+ hybrid plots at two locations. The evidence of this study is that there was no effect of either the inserted gene or the insertion process on the overall performance of the Bt+ hybrid.

CONCLUSION

This paper has described research to define the risk of yield loss due to ECB and the opportunity to reduce that risk with ECB resistant transgenic hybrids. The findings can be summarized as follows: 1) European corn borer can cause yield losses of 5%–10% with only moderate infestation rates and much higher yield reductions are possible with high infestation rates. 2) Damage from ECB is highly variable from year to year and from location to location. Yield losses due to natural infestations ranged from 0 to over 20 percent in the studies described in this paper. Obviously, European corn borer infestations can be a major factor affecting hybrid stability. 3) The

transgenic hybrid described in this paper provided extremely high levels of resistance to ECB. The hybrid showed consistently high relative yields and good physical intactness even with high levels of infestation. The hybrid also had performance equal to the non-transgenic checks in the absence of ECB infestation.

The European corn borer is a costly pest for the American farmer. The image of broken stalks and dropped ears in fields damaged by ECB is only a sign of less obvious, but more important, yield losses. The elimination of these ECB-caused yield losses can improve the yields and the stability of corn production. The image of healthy, intact fields which are resistant to European corn borer is a worthy goal. Insect resistant hybrids developed through biotechnology research offer an opportunity to meet that goal.

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Production and Evaluation of Transgenic Maize Plants Resistant to European Corn Borer

Charles L. Armstrong[1]
Monsanto Company
700 Chesterfield Parkway North
St. Louis, MO 63198

INTRODUCTION

The ability to reproducibly transform corn has greatly broadened the germplasm available for its genetic improvement (Fromm, 1990; Fromm et al., 1990; Gordon-Kamm et al., 1990; Walters et al., 1992; D'Halluin et al., 1992; Omirulleh et al., 1993). One of the first commercial applications targeted for this new technology is the development of corn hybrids resistant to attack by certain lepidopteran insects through the stable introduction and expression of genes encoding insecticidal proteins from *Bacillus thuringiensis* (Bt; Lambert and Peferoen, 1992; Feitelson et al., 1992). Control of the economically important pest, European corn borer (ECB; *Ostrinia nubilalis*), through expression of *cryIA* genes of *B. thuringiensis* has recently been reported (Barnason et al., 1991; Koziel et al., 1993). In addition to the development of maize transformation technology, breakthroughs in obtaining high-level expression of foreign genes in plants were

CHARLES L. ARMSTRONG

[1] Co-authors G.B. Parker, J.C. Pershing, A. Blasingame, S.M. Brown, T. Coombe, D.L. DeBoer, D.A. Dean, B.K. Dennehey, D.R. Duncan, J. Gregory, B.M. Hairston, J. Hart, A.R. Howe, R. Lewis, F.M. Morrish, T.K. Nehet, M.E. Pajeau, W.L. Petersen, B.J. Reich, C. Reich, R. Rodriguez, P.R. Sanders, C.G. Santino, S.J. Sat[illegible] A. Schuler, S.R. Sims, S. Stehling, T. Stone, F.C. Tamayo, L. Tarochione, T.H. Thornton, M.J. Walker, M.E. Ta[illegible]on

CHAPTER 11

Environmental Safety Data

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11. ENVIRONMENTAL SAFETY DATA

A. Environmental Toxicology Studies: Effect of CryIA(b) Protein on Nontarget Organisms

The toxicity of the Bt maize CryIA(b) protein to nontarget organisms was examined. Two primary test materials were used in these studies: (1) a CryIA(b)-enriched leaf protein preparation (referred to as *Bt* maize protein), obtained by extracting *Bt* maize leaves, enriching the protein for the CryIA(b) fraction, and lyophilizing the material to yield a fine protein powder, and (2) pollen collected from *Bt* maize plants (referred to as *Bt* pollen) that were homozygous for the transgenes. In addition, for certain tests comparing the activity of *Bt* maize protein and native CryIA(b), a cell paste containing the CryIA(b) crystal protein produced by fermentation of *B.t.k.* strain HD1-9 was used. This was referred to as native CryIA(b). The specific material selected for a study was based on the most likely route of exposure for the organism being tested (e.g., aquatic organisms were exposed to pollen because that is the most likely part of a corn plant expressing CryIA(b) to enter an aquatic environment).

In addition to testing for potential effects of the transgenic plant products against nontarget organisms by comparison to negative control groups, nontransgenic maize controls were also used in most of the ecological effects studies. These controls consisted of the same test material (i.e., pollen or maize protein) produced by isogenic (nontransformed) maize plants grown under the same environmental conditions as the transformed maize. By including these controls, effects of the test substance *per se* could be distinguished from effects attributed to the presence of CryIA(b) in the test materials from transformed maize.

- **Avian Oral Study on Bobwhite Quail (*Colinus virginianus*) Using CryIA(b)-Enriched Maize Leaf Protein**

  Wildlife International Ltd. (Commerce, MD) conducted an avian acute oral toxicity study using *Bt* maize protein and isogenic maize protein on 8-week old bobwhite quail, according to EPA Guideline No. 71-1. Birds (average body weight of 134 g) were given a single oral dose and observed for 14 days. Thirty birds were divided into three groups of ten birds each. Each group consisted of five males and five females. One group served as a negative control, one group served as an isogenic (nontransgenic) protein control, and one group served as the *Bt* maize protein treatment group. The CryIA(b) birds were dosed with 2000 mg/kg of total protein (containing 0.07% CryIA(b); equivalent to 1.4 mg CryIA(b) protein/kg). This limit dose of total protein was selected after a seven-day rangefinder study conducted at 2000 mg total isogenic protein/kg revealed no signs of toxicity or abnormal behavior (such as an adverse reaction to the protein dosage). The birds in the definitive study were observed at least twice daily during the study for mortality, signs of toxicity, and abnormal behavior. Individual body weights and feed consumption were also recorded during the study.

  No mortalities occurred in any test or control group during the 14-day observation period. There were no remarkable necropsy findings in any test or control birds. No adverse effects on body weight or feed consumption were noted for the birds dosed with CryIA(b) protein when compared to either the isogenic or negative control birds. Based on these results, the acute oral LD_{50} was established to be > 2000 mg *Bt* maize protein/kg (1.4 mg CryIA(b)/kg) and the NOEL was 2000 mg total protein/kg (1.4 mg CryIA(b) protein/kg).

- **48-Hour Static Renewal Toxicity of *Bt* Maize Pollen to *Daphnia magna***

  Springborn Laboratories, Inc. (Wareham, Mass) conducted a 48-hour static-renewal test with *Bt* maize pollen (homozygous for the *cryIA(b)* gene) and isogenic pollen on *Daphnia magna*, according to EPA GuidelineNo. 72-2. Daphnids were <24 hours old at the time of study initiation. For the definitive test, dose levels of 19, 32, 54, 90, and 150 mg pollen/L (containing 5.87 mg CryIA(b)/g pollen) were employed. In addition, isogenic controls at the same pollen concentrations as the treatment group were tested along with a negative control group. Each test or control concentration consisted of two replicates of 10 daphnids each for a total of 20 daphnids/ concentration or control group. Daphnids were exposed for 48 hours with complete renewal of the test solutions after 24 hours.

  Mean survival was 100 percent for each of the transgenic, isogenic, and negative control groups. All daphnids in the transgenic, isogenic, and negative control groups appeared normal during the study. No immobilization or sublethal signs of toxicity were observed. The only effect noted was a decrease in dissolved oxygen in the higher test concentrations of both pollen groups. Dissolved oxygen concentrations were inversely related to the concentration of pollen tested and were similar in equivalent concentrations of the transgenic and isogenic groups. The decrease in dissolved oxygen had no effect on the survival of the daphnids. Higher concentrations for both types of pollen were cloudy and some daphnids were observed to be coated with pollen. At 48 hours, the EC_{50} based on immobilization was >150 mg pollen/L for both the transgenic and isogenic groups. Based on these results, the NOEC was 150 mg transgenic or isogenic pollen/L (the highest concentration tested).

- **Single Dose Test Evaluating Toxicity of *Bt* Maize Protein to Earthworms (*Eisenia foetida*)**

  Springborn Laboratories, Inc. (Wareham Mass) completed a 14-day study on the toxicity of *Bt* maize protein to earthworms based upon OECD No. 207 earthworm testing guidelines. Test groups were exposed to *Bt* maize protein [0.07% CryIA(b)], isogenic (non-transformed) leaf protein, or represented a negative control group. A preliminary 14-day study was conducted at 455, 90.9, and 18.2 mg total leaf protein/kg soil. There were no adverse effects on growth or survival of worms at any concentration in the pilot. Based on these results, a single high concentration of 500 mg *Bt* maize protein/kg soil (0.35 mg CryIA(b) protein/kg soil) was selected for the definitive study. This concentration is equivalent to 1325X the typical environmental exposure if one acre of senescing plants (25,000) were to be incorporated into the soil, or 32X the typical environmental exposure if the same number of plants at peak CryIA(b) production (anthesis stage) were to be incorporated into the soil. An isogenic control at 500 mg maize protein/kg soil was used in addition to a negative control. Each test or control group consisted of four replicates containing ten worms per replicate (40 worms/ concentration). Observations for mortality, toxicity, and behavior were made on day 7 and day 14. Earthworm body weight was recorded on days 0 and 14. A reference test using chloroacetamide was also used to verify the health of the earthworm culture and the proper sensitivity of the test design.

  No effects on survival or signs of toxicity were noted in the worms exposed to transgenic protein, isogenic protein, or in the negative controls on the day 7

observations. After 14 days, no mortalities or signs of toxicity were noted in either maize protein group or the negative control. No adverse effects on earthworm body weight after exposure to transgenic protein as compared to worms exposed to isogenic protein or the negative controls occurred. Results of concurrent testing with chloroacetamide verified satisfactory performance of the test design. Based on these results, the LC_{50} for transgenic protein was determined to be >500 mg protein/kg soil (>0.35 mg CryIA(b) protein/kg soil) and the NOEC was 500 mg protein/kg soil (0.35 mg CryIA(b)/kg soil).

- **The Effect of *Bt* Maize Pollen on Lady Beetle (*Coleomegilla maculata*) Larval Development**

A study assessing the toxicity of *Bt* maize pollen (homozygous for the *cryIA(b)* gene) to lady beetle larval development was conducted at Iowa State University (Ames, IA), according to EPA Guideline No. 154A-23. *C. maculata* larvae were reared on either transgenic maize pollen, isogenic (control) pollen, or an optimal diet of pea aphids. No other food source was available to the lady beetles during the study. Testing was initiated with first instar larvae and these were followed until adult emergence of survivors. Prior to the initiation of the definitive study, a pilot study using hybrid *Bt* corn pollen, which was hemizygous for the transgenes, was conducted. In this pilot study, five out of five *C. maculata* larvae successfully completed development to adults. In the definitive study there were three replicates of 15 larvae/replicate in each test or control group. Larval survival, development times of the different life stages, and adult weight were measured.

Results of the definitive study indicated that survival of *C. maculata* larvae reared on pea aphids was 91%, survival of larvae raised on isogenic control pollen was 43%, and survival of larvae raised on transgenic pollen was 47%. Pea aphids are an insect prey species considered to be an optimal diet for *C. maculata* development and the good survival observed for this group indicates that appropriate environmental conditions for larval development were maintained during the test. The pattern of mortality observed in the two pollen groups was similar, with most mortalities occurring in the later developmental stages (fourth instar through eclosing adult life stages). The observed mortalities were not attributed to the presence of CryIA(b) in the transgenic pollen group because of similar effects in the isogenic group; rather it is suspected that the pollen (transgenic and isogenic) may not have provided sufficient nutritional factors for optimal development. Development time in both pollen diet groups was significantly longer in first through fourth instars compared to development time in the pea aphid diet group. There was no significant difference in development time between the two pollen groups. There was no significant difference among the three treatments in pupal development times. The mean weight of emerged adults in the isogenic pollen diet group was significantly less than the mean weight of emerged adults in the transgenic pollen and pea aphid diet groups. There was no significant difference in the weight of adults raised on transgenic pollen compared to those raised on pea aphids.

- **The Effect of Bt Maize Pollen on Larval Honeybee (*Apis mellifera* L.) Development**

Pollen produced by event 176-derived maize plants contains CryIA(b) protein, and ingestion of this genetically modified pollen is anticipated to be the primary route by which honeybees will be exposed to Bt maize. A study conducted by California

Agricultural Research (Kerman, CA), evaluated whether ingestion of Bt maize pollen from event 176-derived maize plants had any measurable effects on larval honeybees (*Apis mellifera* L.) maturing within honeycomb brood cells.

A single dose study was conducted in which approximately 1 mg of *Bt* maize pollen and a drop of water was administered into the diet of three to five day old honeybee larvae. For comparative purposes, one control treatment included larval bees that received no treatment while a second control group was administered a 1 mg dose of non-transgenic pollen and a drop of water. A positive control involved the incorporation of the carbaryl insecticide Sevin® and non-transgenic pollen into the larvae's diet. Each treatment included four replicates of 25 larvae each. Following treatment, the bees were allowed to be capped inside their source hives and later moved to a growth chamber. Those bees surviving to emergence were counted and all treatments were statistically compared to ascertain treatment effects on larval bee survival as well as time to adult emergence.

Three statistically significant groups were identified: (1) larval bees administered the genetically modified pollen had an average emergence frequency of 95%, while the untreated group's value was 96%, (2) a 65% emergence frequency from the group receiving non-transgenic pollen, and (3) 4% emergence from the carbaryl treated group. The cause of the reduced emergence frequency in the non-transgenic pollen group is unclear. Relative differences in hive vigor or genetic variability may have contributed. There were no differences observed among any of the groups in the average number of days to emergence, nor were any behavioral effects observed.

Based upon these results, there are no measurable detrimental effects of ingestion of CryIA(b)-containing pollen on larval honeybee development.

- **Insect Target Comparison of Native and Maize Expressed CryIA(b) Protein**

Ricerca, Inc., Painesville, OH, conducted a study bio-assaying four species susceptible to native *Bt* CryIA(b) (European corn borer (ECB), corn earworm, cabbage looper, and Southwestern corn borer) and two species not susceptible to native CryIA(b) (fall armyworm and black cutworm). Assays using neonate larvae were conducted using *Bt* maize leaf protein and native, full length CryIA(b) protein. Comparative assays using both types of *Bt* protein were conducted three times for each species using 30 individuals in each test group. Multiple doses were used; the test materials were applied to the surface of standard insect diets for each species. Mortality was assessed after a four day feeding period and a LC_{50} for each type of test material was determined for each susceptible species. A single, high dose ($\geq$ the European corn borer LC_{90}) was used for testing the non-susceptible species.

Results indicated that the maize *Bt* protein, when expressed as ng CryIA(b)/cm^2 diet surface, was more potent than the native CryIA(b). This is accounted for by the fact that the maize CryIA(b) polypeptide is a truncated version of the native protein, so there is more active endotoxin per unit weight of protein. The relative sensitivity among three of the four susceptible species remained the same for native CryIA(b) and maize CryIA(b); no conclusions could be drawn from the tests with maize protein on Southwestern cornborer because of apparent feeding inhibition by the *Bt* maize leaf material as well as the control maize leaf protein preparations. The two non-susceptible species (fall armyworm and black cutworm) were not affected by exposure to doses of each test material $\geq$ the ECB LC_{90}. These results indicate that

the truncation of the native CryIA(b) protein to the maize CryIA(b) polypeptide did not affect the insect target specificity of the CryIA(b) polypeptide in comparison to its native, full-length counterpart.

- **Impact of Transgenic Maize Expressing Truncated CryIA(b) Protein on Nontarget Insect Populations**

  Ciba Seeds conducted a small plot field study in Bloomington, IL during the summer of 1993 to evaluate the impact of maize expressing the CryIA(b) endotoxin on associated populations of insects. The study focused on beneficial predators and parasites in the orders Diptera, Hymenoptera, and Coleoptera (Coccinellid family) as well as Homopterans, which represent an important food source for beneficial predators. Insect populations in transgenic hybrid maize plots were compared to populations in isogenic hybrid maize and wild type maize plots. The study also evaluated the impact of a conventional chemical insecticide, permethrin, on insect populations in maize. There were three replicate plots of each type of treatment. Insect populations were monitored weekly over a 10 week period from mid-June through early September using Scentry Multigard® yellow sticky traps. Two traps were placed in each plot (plots were approximately 7 m long by 3 m wide). Traps were coded at collection and sent to an independent laboratory (Ricerca, Inc) for scoring.

  Results of the monitoring study indicated no difference in the number of total insects or the numbers in specific Orders between the transgenic maize plots and either the isogenic or wild type control maize plots. There was no shift in the taxonomic distribution of insects associated with the *Bt* maize compared to the control maize. In contrast, treatment with permethrin had significant effects on the total numbers of insects and on the numbers within specific groups compared to the untreated plots. The beneficial lady beetle predators (coccinellids) were particularly susceptible to permethrin. Coccinellids, dipterans, and hymenopterans represent the majority of beneficial predators and parasites associated with maize. The results of this monitoring study suggest that expression of CryIA(b) in maize should not adversely effect insects in these groups.

Summary of Environmental Toxicology Studies

In all of the studies outlined above, there is no evidence that exposure to the CryIA(b) protein expressed in either maize pollen or extracted from *Bt* maize leaves resulted in any toxic effect on the organism tested. Tested organisms include representative avian, aquatic invertebrates, and soil invertebrate species, and several nontarget insect species. Testing with insects known to be either susceptible or not susceptible to native CryIA(b) gave no indications of a changed host specificity for the maize expressed CryIA(b). A small field monitoring study on beneficial insects did not detect any effects on beneficial insect or insect prey species exposed to *Bt* maize. These results suggest that only lepidopterans susceptible to native CryIA(b) are likely to be affected by *Bt* maize CryIA(b).

B. Endangered Species Considerations

Endangered lepidopterans may conceivably be sensitive to CryIA(b) protein, given that the protein is selectively toxic to certain lepidopterous species. Endangered lepidopterans

include several species of moths and butterflies: the El Segundo blue butterfly, the primrose sphinx moth, Lange's metalmark butterfly, the Lotis blue butterfly, the Oregon silverspot butterfly, the San Bruno elfin butterfly, Schaus' swallowtail butterfly, and Smith's blue butterfly. However, the potential for exposure of these species to CryIA(b) via *Bt* maize is virtually nonexistent. None of these species is found in agricultural areas where maize (corn) is grown, and maize is not among the host plants for these lepidopterans. Unlike the exposure scenarios typical of conventional or microbial insecticides, an organism must actually consume maize tissue to receive any exposure to CryIA(b). Host-range comparisons have not indicated any change in range of species susceptible to maize CryIA(b) compared to native CryIA(b). Both field testing and laboratory testing of maize CryIA(b) have indicated that nontarget beneficial insects are not likely to be affected by maize CryIA(b), so it is not likely that endangered dipterans, hymenopterans, or coleopterans would be affected.

Another possible, though improbable, impact on endangered species in other taxonomic groups, such as birds, is through changes in food supply. However, European corn borer (ECB), the target insect for CryIA(b) in maize, is not a significant food source for birds. As a cryptic insect, ECB larvae bore into the plant and become inaccessible to predators. Although beneficial lady beetles feed on ECB eggs, the potentially reduced ECB populations in areas where *Bt* corn is grown should not impact this predator species because it has many other food sources. Moreover, ECB feeds upon several different crop plants and vegetables. Currently available data suggest that the presence of CryIA(b) protein in maize will not significantly impact either the total number or taxonomic distribution of insects associated with maize. The target organism specificity of CryIA(b) indicates that only a specific group of lepidopterans should be affected, with the majority of potential prey species remaining unaffected. Potential exposure of endangered aquatic organisms would primarily be through pollen blown into the water. Available data on aquatic invertebrates indicates that CryIA(b) in pollen should not pose a risk beyond that from pollen alone, which may affect dissolved oxygen if the concentration becomes sufficiently high.

C. Potential for Outcrossing of *cryIA(b)* and *bar* Genes to Other Plants and Organisms

The following is an excerpt from USDA APHIS, Environmental Asessment 92-042-01, pages 6-9, by Dr. James Lackey. It is provided as background for our discussion on event 176.

- "Genes of corn may escape from the test plot in two ways. The first is by pollen transfer. The second is by movement of the grains.

  If viable pollen of the transgenic plants can be transferred by wind to any receptive corn stigma genetic material could take place. Movement of corn pollen by wind is limited in distance due to its large size and rapid settling rate (Raynor et al., 972). The distance the female is from the male source also greatly influences the frequency of outcrossing. Research has shown that relatively short distances from the source of contaminating pollen can reduce outcrossing to less than 1% percent (Paterniani and Stort, 1974). Another study has shown that a distance of 20 feet can reduce outcrossing below the minimum standard for certification (Hutchcroft, 1959). Therefore, potential transfer becomes more unlikely as distance increases from the transgenic plants, and from a practical standpoint becomes increasingly unlikely at distances much beyond the foundation seed isolation distance of 660 feet.

Temporal isolation would further reduce the likelihood of effective pollination and fertilization. In addition, any physical impediment to this movement, such as effective detasseling or bagging, would completely eliminate the possibility of gene escape by way of pollen.

To prevent grain from remaining in the field or otherwise escaping, all ears would have to be collected or otherwise destroyed. To ensure that no grain escaped harvest, the field would have to be monitored for volunteer corn plants in the following season."

- **Outcrossing with Wild Species.** There are no wild, weedy relatives of Zea mays known to exist in the United States. Outcrossing of the *cryIA(b)* or *bar* genes, therefore, does not pose a plant pest risk due to the enhancement of weediness of wild relatives of corn.

- **Outcrossing to Cultivated Corn and the Weediness Potential of BT Corn.** As described in Dr. Lackey's excerpt, the potential for outcrossing of the *cryIA(b)* or *bar* genes to non-Bt corn exists. However, the frequency at which this event would occur is expected to be very low due to the short distances that corn pollen is transferred and its very limited window of viability. In addition, the potential for outcrossing to occur during seed production is further reduced due to traditional containment practices to ensure seed genetic purity. The physical isolation distance for transgenic corn environmental releases under USDA permits or notification is based on standard seed purity isolation practices.

  Upon commercial introduction Bt corn may be grown in close proximity to non-Bt corn, allowing for potential outcrossing of the transgenes to nontransgenic corn. This would be inconsequential due to the negligible amounts of transgenic proteins that would be present in the seed progeny of such outcrosses. Moreover, such seed would not likely be planted back because virtually all commercial corn not grown for seed production is grown from hybrid seed purchased from seed companies.

  The potential impact of commercial introduction of Bt corn on the production of non-hybrid seed grown for consumption was considered. The Seeds Saver Exchange (Decorah, IA), The Seeds Science Center (Iowa State University), and the Economic Research Service at the National Agricultural Library (Washington, D.C.) were consulted to determine such factors as number of acres grown, their location, and mode of production. The consensus from these sources is: in the United States, non-hybrid seed is not grown or used for consumption purposes. While there may be a few "hobby" growers, no statistics are maintained and these references believe that the amount of non-hybrid maize grown in the United States for consumptive purposes is less than one acre.

  While the transgenes could be expressed in volunteer corn, the weediness potential of such plants will not be greater than that for nontransgenic volunteer corn. Corn is a highly domesticated crop and has never been able to establish itself outside of cultivated areas. Resistance to European corn borer and the glufosinate herbicide (not currently registered for use on corn in the US) are but two traits that, although significant to the farmer, do not impact a corn plant's ability to survive without human intervention. Such plants will be easily controlled by traditional methods of controlling volunteers, such as physical cultivation or application of registered

herbicides active against corn. Observations of field trials at several sites during the past two years indicate that Bt corn volunteers occur in fields at the same rate as non Bt corn and are just as susceptible to traditional control practices. Additional data concerning this aspect will be presented in the Agronomic and Compositional Evaluation Chapter in this petition.

- **Transfer of Genetic Information to Organisms with which Corn Cannot Interbreed.** The USDA's Interpretive Ruling on the Calgene, Inc., "Petition for Determination of Regulatory Status" (FR 57, No. 202, pp 47608-47616) states that "there is no published evidence for the existence of any mechanism, other than sexual crossing" by which genes can be transferred from a plant to other organisms.

  Limited DNA homologies between some plant and microbial genes are consistent with but do not prove gene transfer from plants to microorganisms in evolutionary time over many millennia. Such homologies are expected in the absence of gene transfer through normal evolutionary mechanisms.

  Escape of transgenes from maize to microbes is a less likely route of acquisition than from other microbes. Since microbes have developed specific (transduction, conjugation) and non-specific (transformation) systems for DNA exchange between species and genera, it is plausible that DNA transfer would occur among bacteria with much greater likelihood than it would take place between plants and bacteria. Yet even between bacteria gene transfer is a highly controlled process. For example, endotoxin genes which are readily exchanged between species of *Bacillus thuringiensis* and *Bacillus cereus* are not found in other bacteria. This is due to the fact that microbes have evolved elaborate systems to protect their genomes from the non-discriminate introduction of genes from both related and non-related organisms. Rarely does gene transfer occur between distantly related organisms. When it does occur, as in the case of the transfer of *Agrobacterium* DNA to plants, the process is complex, highly specific and involves many temporally regulated genes.

  However, even if such gene transfer were to occur with respect to Bt corn, the recipient microbes would acquire genes that originated from non-pest microbes. Such recipients would therefore not pose a greater plant pest risk than the wild type microbes from which the genes originated. Based on these considerations, plant to microorganism genetic transfer of the *cryIA(b)* and *bar* genes is of no significant consequence from an environmental or plant pest perspective.

STATEMENT OF GROUNDS UNFAVORABLE

Ciba Seeds Is Unaware At This Time Of Any Conditions That Are Unfavorable To This Request For Nonregulated Status of Ciba Seeds *Bt* Corn

REFERENCE DOCUMENT I

ASSESSMENT AND MANAGEMENT OF EUROPEAN CORN BORER RESISTANCE TO TRANSGENIC CORN (MAIZE) CONTAINING THE EVENT 176 INSECT CONTROL PROTEIN (EPA MRID No. 43415704)

submitted by

Ciba Seeds
Ciba-Geigy Corporation
3054 Cornwallis Road
Research Triangle Park, NC 27709-2257

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VOLUME 1 OF 1 OF SUBMISSION

ASSESSMENT AND MANAGEMENT OF EUROPEAN CORN BORER RESISTANCE TO TRANSGENIC CORN (MAIZE) CONTAINING THE EVENT 176 INSECT CONTROL PROTEIN

EPA GUIDELINE #: NOT APPLICABLE

AUTHOR:

DEAN CHRISTENSEN
CIBA SEEDS

SUBMITTED ON: 18 OCTOBER 1994

SUBMITTED BY:

CIBA SEEDS
CIBA-GEIGY CORPORATION
POST OFFICE BOX 12257
RESEARCH TRIANGLE PARK
NC, USA 27709-2257

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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

Company: Ciba-Geigy Corporation

Company Agent: Demetra Vlachos Date: October 18, 1994

Title: Manager, Biotechnology Regulatory Affairs

Signature: Demetra Vlachos

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

Good Laboratory Practice standards do not apply to the information contained in this volume. Therefore, certification of Good Laboratory Practice compliance as described in 40 CFR Part 160 is not applicable.

Submitted by:

Demetra Vlachos
Manager, Biotechnology Regulatory Affairs
Ciba Seeds

October 18, 1994
Date

Submitter/Sponsor:

Ciba Seeds
Ciba-Geigy Corporation
Post Office Box 12257
Research Triangle Park, NC 27709-2257

CIBA SEEDS

ASSESSMENT AND MANAGEMENT OF EUROPEAN CORN BORER RESISTANCE TO TRANSGENIC CORN (MAIZE) CONTAINING THE EVENT 176 INSECT CONTROL PROTEIN

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CIBA SEEDS

ASSESSMENT AND MANAGEMENT OF EUROPEAN CORN BORER RESISTANCE TO TRANSGENIC CORN (MAIZE) CONTAINING THE EVENT 176 INSECT CONTROL PROTEIN

October 18, 1994

Summary

Ciba Seeds corn transformation event 176 produces a truncated version of the CryIA(b) protein from *Bacillus thuringiensis (Bt)*. Corn (maize) that produces the event 176 insect control protein (176 ICP) is protected from damage caused by the European corn borer (ECB) during the major portion of the growing season. ECB has never developed resistance to a Bt protein. However, in some isolated cases, other lepidopteran insects have developed resistance to Bt microbial insecticides. Thus, there may be a possibility that ECB could become resistant to the 176 ICP.

The risk due to potential ECB resistance to 176 ICP is a product of the effects of resistance and the probability of its occurrence. Overall, the effects of ECB resistance are low to moderate, and primarily limited to the agricultural community. There is little likelihood that 176 ICP resistance could enable ECB to invade new environmental niches. For growers and consumers, ECB resistance to 176 ICP could result in a return to the current cost situation for ECB damage and control options in corn. The efficacy of current commercial Bt microbial products is not likely to be affected by ECB resistance to 176 ICP unless there is significant cross-resistance to the multiple Bt insect control proteins in these products.

The following points are relevant in assessing the probability for occurrence of ECB resistant to 176 ICP: 1) ECB has not developed resistance to Bt microbial products, although this has occurred for several other lepidopteran species, 2) only rare individuals are expected to survive exposure to 176 ICP, 3) ECB are mobile and can mate with individuals from nearby fields, and 4) during at least the first four years following commercialization of 176 ICP corn, there will be ample numbers of susceptible ECB from conventional corn fields to prevent fixation of any rare alleles for resistance. The probability of ECB resistance to 176 ICP corn within the first several years of commercial sales is low due to high

efficacy of the product and the maintenance of susceptible genotypes within the ECB population.

The combination of a low to moderate effect with low probability of occurrence results in only a low risk due to 176 ICP resistance during at least the first four years of commercialization.

Even though the likelihood of resistance is low, Ciba Seeds is planning to minimize any possibility of ECB resistance to 176 ICP. Numerous theoretical options have been suggested to mitigate the potential risk arising from ECB resistance, but little is known concerning the efficacy of most of the choices. Nevertheless, Ciba has implemented strategies to 1) develop genes for new insect control proteins, 2) monitor ECB susceptibility to 176 ICP, and 3) conduct research in key topics relevant to ECB resistance management. Ciba Seeds' resistance management strategies will be adjusted as additional information is obtained from this and other research.

Introduction

Ciba Seeds has produced transgenic corn that expresses a truncated version of the CryIA(b) protein from *Bacillus thuringiensis* (Bt) subsp. *kurstaki* strain HD1 (Koziel, et al. 1993a). Progeny of one of these transformation events, named event 176, have been shown to provide a high level of protection from damage caused by the European corn borer (ECB) (*Ostrinia nubilalis)* (Koziel, et al. 1993a; Christensen, et al. 1993). Ciba Seeds has applied for regulatory approval to market corn hybrids derived from event 176. However, the benefit of these products to growers will be reduced if ECB develops resistance and is able to cause economic damage to corn plants that contain the insect control protein produced by event 176 (176 ICP). The purpose of this document is to assess the potential risk posed by ECB that are resistant to 176 ICP, evaluate options to mitigate this risk and outline resistance management strategies for Ciba Seeds.

Ciba Seeds is aware of the potential problems of ECB resistance to Bt insect control proteins. Ciba has participated in the Bt Management Working Group, an industry consortium, to address this issue and sponsor relevant research. Ciba Seeds has also conducted internal research and sponsored independent research on ECB resistance topics. In preparation for this document, an internal ECB resistance management group, containing representatives from diverse scientific backgrounds, was formed to formulate policies and coordinate research activities. This group requested the advice of a panel of public researchers (Appendix 1) via a questionnaire on ECB resistance topics (Appendix 2). Responses to the questionnaire were important in formulating this paper. Complete copies of the responses are available on request.

The discussion of ECB resistance in this paper is divided into five sections. First, the potential effects of ECB resistance to 176 ICP are defined for the environment, growers and consumers. Second, the probability of occurrence of ECB resistance is discussed. Third, the overall risk of ECB resistance is estimated as a product of the

effects and their probability of occurrence. Fourth, options to mitigate the risk of ECB resistance are discussed and evaluated. Finally, Ciba Seeds' resistance management strategy for corn containing 176 ICP is described for initial commercialization and the mid-term future.

Potential Effects of ECB Resistance to 176 ICP

Overall Assessment of Effects of Resistance

The effects of ECB resistance to 176 ICP are low to moderate, and primarily limited to the agricultural community. There is little likelihood that ECB that are resistant to 176 ICP could invade new environmental niches. For growers and consumers, ECB resistance to 176 ICP could result in a return to the current cost situation for ECB damage and control options in corn. The efficacy of current commercial Bt microbial products is not likely to be affected by ECB resistance to 176 ICP.

Environment

If ECB develop resistance to 176 ICP, there appears to be little likelihood that the fitness of the resistant insects could be altered such that damage to crop and non-crop environments is increased. Tabashnik (1994) reviewed research on the stability and fitness of colonies of insects other than ECB with resistance to Bt insect control proteins. The insects in these studies showed either reduced or neutral fitness in the absence of selection for Bt insect control protein. ECB that are resistant to 176 ICP have not been identified, and there is no direct research data concerning the fitness of such ECB in the absence of 176 ICP selection. However, there is no obvious fitness advantage for ECB that are resistant to 176 ICP.

Growers and Consumers

The most likely effect of potential ECB resistance to 176 ICP could be a return to the current situation for ECB damage and control options. The environmental and economic benefits of these products to growers and consumers could be reduced or lost. Corn growers could either not control ECB damage or practice

the same control methods used today. Applications of conventional insecticides for ECB control are less efficient than plants with 176 ICP, in part due to the restricted time period in which they can be effectively applied.

Damage caused by ECB to other crops, such as potatoes, peppers or cotton, is usually minor. Growers of these crops could also lose the option, if it exists, for protection from ECB damage with transgenic plants containing CryIA(b) protein.

Commercial Bt microbial products are a mixture of Bt insect control proteins with different sites of action in an insect's gut. CryIA(b) is not a major component of most of these products (internal Ciba data). However, the effectiveness of Bt microbial products could be reduced if ECB that were resistant to 176 ICP were also resistant to other Bt insect control proteins. The available evidence indicates that cross-resistance, if it occurs, is likely to only affect Bt proteins with the same insect gut binding site as CryIA(b) (note discussion of cross-resistance in following section). Also, since Bt microbials are currently used on less than 5% of the corn acres, this worst-case effect of resistance on ECB control in corn would be small, relative to the current situation.

Probability of ECB Resistance to 176 ICP

Overall Assessment of Probability

The probability of ECB resistance to 176 ICP can only be incompletely assessed. Plants that produce 176 ICP have only recently been available for research projects, and very little is known about the genetics and behavior of ECB related to resistance. Efficacious levels of 176 ICP are produced in critical tissues of corn plants through the major portion of the growing season, resulting in a very high level of protection from ECB damage. Despite this, the possibility exists that ECB resistance to 176 ICP corn could occur, given the appropriate selection environment. These selection environments are not likely to exist during at least the first four years of commercialization. Thus, the probability of ECB resistance to 176 ICP must be assessed to be very low during the first several years following commercialization.

Documented Insect Resistance to Bt Insect Control Proteins

Tabashnik (1994) reviewed reports of resistance to Bt insect control proteins in five lepidopteran and two coleopteran species. All but one of the resistant species were predominately selected in the laboratory. Laboratory selection experiments may provide useful information about the presence of alleles for resistance, but they likely have limited relevance for assessing the probability of field resistance, especially to transgenic plants. There are two major reasons for this conclusion. First, laboratory experiments have generally been conducted

with a very small, genetically isolated subset of a field population. As Koziel (1993b) noted, rare alleles that could confer a high level of resistance might be missing from this sample. Second, individuals in these selection experiments have generally been subjected to acute (short-term) sublethal selection for numerous generations. This procedure has favored the accumulation of polygenic resistance factors. In contrast, Ciba Seeds' transgenic corn plants produce high levels of 176 ICP relative to the LC_{50} for ECB through the major portion of the growing season. It is highly unlikely that quantitative resistance to these plants could develop. In fact, it is most probable that only a very rare individual, homozygous for a single major gene conferring a high level of resistance, could survive on plants that are actively expressing 176 ICP (see following discussions of gene action and selection pressure).

The example of the diamondback moth (*Plutella xylostella)* populations that have developed moderate to high resistance to Bt insect control proteins in the field is useful. Koziel, et al. (1993b) outlined several factors concerning resistance in this insect that are relevant for assessing the probability of resistance to transgenic 176 ICP plants. First, resistance generally developed in geographically isolated populations that had little immigration. Thus, resistant genotypes were likely to mate with each other and increase the frequency of resistance alleles in the population. Second, populations produced large numbers of generations per year, which again increased the possibility for fixation of resistant genotypes. Third, populations were treated continuously with a single Bt microbial product. Insects were under cyclical selection pressure because the efficacy of Bt microbials declines rapidly after application. These periods of sublethal selection may have favored survival of some resistant insects.

In comparison, ECB are fairly mobile and produce only one to three generations per year. As noted above, corn with 176 ICP provides a high level of protection from damage during the periods when most ECB infestations occur. These factors decrease the probability of ECB resistance to 176 ICP.

Genetic Variables Affecting Resistance Development

1. Resistance gene frequency

Gene frequencies for resistance to 176 ICP are unknown for ECB because resistant individuals have not yet been identified. Researchers surveyed by Ciba Seeds estimated resistance gene frequencies to be between 10^{-3} and 10^{-5}. Given these frequencies, Evola (1994) presented calculations of the potential number of resistant ECB in Illinois, assuming that the resistance allele is recessive, the ECB population is 1.2 larvae/corn plant and that there are 10^7 acres of corn in the state:

Frequency of Resistance Allele	Number of Resistant ECB in Illinois
10^{-3}	380,000
10^{-4}	3800
10^{-5}	38

The probability of establishment of an ECB population with fixed resistance alleles is obviously dependent on the frequency of those alleles. If the frequency of resistance alleles is 10^{-5}, the probability of fixation of resistance alleles will be very low. On the other hand, if the frequency is 10^{-3}, resistance may be measurable, and resistance management strategies might be needed.

2. Resistance gene action

This variable is also unknown for ECB because resistant individuals have not been identified in the field. Tabashnik (1994) reviewed genetic research with resistant populations of other insects. Most reports documented partial to completely recessive gene action. Based on existing data, it might be predicted that any ECB resistance genes for 176 ICP corn would be at least partially recessive and may confer some selective advantage for heterozygotes.

3. Fitness of resistant genotypes

Once again, the effect of this variable is unknown for ECB. As noted in the previous discussion of environmental hazard, studies of other Bt-resistant insects have shown reduced or neutral fitness associated with resistance. If the fitness of resistant genotypes is assumed to be neutral relative to susceptible genotypes, the frequency of resistance alleles will decline only slowly in the absence of selection. On the other hand, the frequency of resistant insects with reduced fitness would be expected to decline more rapidly in the same situation.

4. Gene flow

ECB leave corn fields to mate in neighboring grassy "action sites." They can travel several miles on prevailing winds (Showers, 1993). However, little is known about the actual degree of gene flow from ECB in one field to those in neighboring fields. The degree of intermating is affected by the relative numbers of individuals from each field, weather conditions, and the distance between fields. In the corn belt, corn is the predominant host of ECB, but in the southern and eastern U.S., a significant degree of mating may occur between individuals from corn and from other host crops.

Mating is restricted between the univoltine and multivoltine races of ECB and also between the E and Z pheromone types of ECB. The multivoltine Z race is predominant throughout much of the midwest, so race-related mating restrictions are probably not important in most of the corn belt.

In sum, it can be assumed that although there is gene flow over local areas, most mating occurs between individuals from the same or nearby fields. If fields near a transgenic 176 ICP field are planted with non-transgenic hybrids, there will be a generous supply of non-selected ECB to mate with any surviving individuals from the 176 ICP field. In this situation the probability of fixation of resistance alleles will be low.

5. Selection pressure

Leaf tissue of Ciba Seeds' transgenic plants produce approximately 1400 nanograms of 176 ICP per gram *fresh weight*. The 176 ICP is also produced in pollen of these plants, and recent Ciba data indicates that the pollen 176 ICP level is approximately 1800 nanograms of 176 ICP per gram *pollen*. Ciba research has established the LC_{50} for ECB at 70 nanograms CryIA(b) per gram *diet*. Although direct comparison of these figures is difficult, 176 ICP is obviously produced at very high levels relative to the LC_{50}. Corn leaf tissue with 176 ICP provides nearly 100% protection from first brood ECB damage and at least 95% protection from second brood damage (Koziel, et al. 1993a) The effect of transgenic pollen containing 176 ICP was not included in the data reported by Koziel, et al., so the actual degree of second brood ECB protection in field situations may be even higher than reported. Although no directly applicable research information is available, plants actively expressing the 176 ICP would be expected to control ECB that have alleles conferring slight increases in resistance. Thus, there is a negligible probability that these alleles could accumulate and confer quantitative resistance to 176 ICP in a local population.

Some ECB may be able to escape exposure to the 176 ICP in Ciba Seeds plants. Silks of plants derived from event 176 contain little or no 176 ICP. A small percentage of ECB larvae on heavily infested plants have been observed to survive in the silks, migrate to the lower stalk, bore into the stalk and survive into the winter. Also, the level of 176 ICP declines as corn plants senesce and grain reaches physiological maturity. Observations taken during the 1994 season indicate that ECB larvae which emerge very late in the season can survive on these plants. The degree of selection pressure, if any, exerted on these ECB is being actively investigated by Ciba Seeds.

Given that the selection pressure on larvae in 176 ICP fields is very high, maintenance of susceptible genotypes in the breeding population could be important to avoid fixation of resistance alleles. Some susceptible ECB may survive on off-type corn plants in the field. However, off-type plants comprise only 0-5% of commercial hybrid fields, so very few susceptible ECB are likely to be available from this source. ECB can reproduce on numerous alternate hosts, including weeds, vegetables and grain crops. However, corn is the predominant host of ECB, and in most corn growing regions very little acreage of alternate host plants is available. The best source of susceptible ECB genotypes is likely to be survivors from neighboring fields that do not produce 176 ICP. These

fields will be readily available during at least the first four years following commercialization of 176 ICP corn. Given the improbable situation of unlimited availability, growers are likely to adopt ECB-resistant corn on a maximum of 50-80% of the corn acreage within that time period (Ciba economic benefits analysis, in preparation). Therefore, although selection pressure within 176 ICP fields will be very high, the selection pressure on the total breeding population will be much more moderate, and the probability for fixation of resistance alleles will be low.

Microbial Products That Contain CryIA(b)

Microbial products that contain CryIA(b) could affect the development of ECB that are resistant to 176 ICP. Such products would have to be used in large quantities near significant acreages of 176 ICP corn, and they would have to be used for crops that could be infested by ECB. These conditions are not likely to be met, given both the minimal acreage of alternative ECB host crops and the minimal use of Bt microbial products in major corn growing regions. Therefore, the probability of ECB resistance to 176 ICP will likely not be affected by these products.

Cross-Resistance of ECB to Multiple Bt Insect Control Proteins

The available evidence indicates a low probability for field levels of cross-resistance not related to insect gut binding site modifications. Tabashnik (1994) reviewed relevant research and concluded that if reduced insect gut binding is the mechanism of resistance, cross-resistance could only be expected between insect control proteins that share binding sites. Gould et al. (1992) reported cross resistance in a strain of *Heliothis virescens* that did not appear to be related to binding site modifications. Laboratory selection with CryIA(c) resulted in varying levels of cross-resistance to CryIA(a), CryIA(b), CryIB, and CryIC insect control proteins. However, Ciba research with this strain of *H. virescens* showed it to be controlled by low to moderate levels of CryIA(b) expressed in tobacco and also by the Bt microbial product Agree™, which contains CryIA(c), CryIC and CryID delta endotoxins. Direct evidence is not available for cross-resistance of ECB selected on transgenic 176 ICP corn plants, since no resistant ECB have been identified. However, it is reasonable to assume that if resistance to 176 ICP occurs, some cross-resistance will also occur to insect control proteins that share binding sites with 176 ICP. This type of cross-resistance should be predictable from competitive binding studies.

Assessment of Risk Due to ECB Resistance to 176 ICP

The risk due to ECB resistance to 176 ICP is the product of the effects, or cost, of resistance and the probability of occurrence of that cost. The combination of a low- moderate effects and low probability results in an insignificant risk due to 176 ICP resistance during the first four years of commercialization. The risk beyond this period will be affected by ECB resistance genetics as well as change or maintenance of the pool of susceptible genotypes necessary to prevent fixation of any resistance alleles in the ECB population. Additional research during the next several years on genetic factors of ECB that affect resistance to 176 ICP should aid the development of improved estimates for the probability of resistance.

Options to Mitigate the Risk from ECB Resistance to 176 ICP

Although the immediate risk of ECB resistance to 176 ICP is minimal, Ciba Seeds has evaluated options to reduce the risk. These evaluations will continue as additional research information is obtained. Risk can be reduced either by lowering the effects of resistance or by lowering the probability of occurrence for resistance. Options are listed below for both alternatives.

Reduction of the Effects of ECB Resistance

Develop ECB control plants with modes of action different than 176 ICP
This action will maintain for growers the option of protection from ECB damage. With an appropriate deployment strategy, new ECB control genes may also reduce the probability of ECB resistance to 176 ICP (see below). Because of the benefits of ECB control for growers, development of new ECB control options is a critically important activity within Ciba.

Reduction of the Probability of ECB Resistance

The likelihood of resistance can be lowered with either gene deployment or refugia strategies. The purpose of both sets of activities is to reduce the chance of fixation of resistance alleles in a breeding population of target ECB.

McGaughey and Whalon (1992) outlined possible gene deployment strategies. They listed numerous options, but they also noted a shortage of experimental data assessing the value of potential strategies. This type of comparative research is important for development of a successful resistance management program:

1. Gene deployment strategies

Expression

Low expression of a gene encoding an insect control protein has been proposed as an option to allow a percentage of susceptible genotypes to survive and yet limit crop damage. This option has serious limitations: 1) resistant and partially resistant genotypes will likely reproduce at a higher rate than susceptible ECB, leading to rapid loss of efficacy, 2) the amount of allowable, non-economic crop damage varies by variety, agronomic practices, and environment, and 3) effective use will require additional pest management strategies that depend on voluntary grower participation.

High expression of genes encoding an insect control protein will theoretically eliminate all but rare homozygous resistant individuals. This option is appealing because crop damage is eliminated, but additional strategies might be necessary to prevent mating of rare resistant genotypes and subsequent development of a resistant population.

Constitutive expression provides Bt insect control protein in all tissues of the plant. Given adequate expression, no part of the plant should allow survival of ECB.

Tissue-specific expression could theoretically kill ECB in economically important tissues but allow survival of susceptible genotypes in other parts of the plant. However, current technology usually allows only tissue-preferential expression, but not tissue-specific expression. Low expression in some tissues could allow sub-lethal selection for resistant ECB. On the other hand, tissue-specific promoters for multiple tissues can be used to provide high Bt insect control protein levels in all critical parts of the plant.

Inducible expression could be "turned on" by feeding damage or chemical application. However, wound-inducible expression could allow economic damage before a Bt insect control protein reached a controlling level. Chemically inducible expression would require additional grower resources for scouting, purchase and application of chemical.

Multiple Genes

Mixtures of gene products can be formed by pyramiding the genes in one plant or by physically mixing seeds with individual genes. Theoretically, ECB resistance is delayed because of the multiple sources of mortality. However, McGaughey and Whalon (1992) cite computer modeling data that shows mixtures may not be better than sequential use of individual insect control proteins. More research is clearly needed to evaluate these strategies.

Rotational or sequential use of individual gene products that target different receptors in ECB could delay resistance by changing the selection pressure on the ECB population. This option is appealing because it could be convenient for seed companies to introduce different transgenic products as improved seed products are brought to the market. However, efficient rotation of insect control proteins depends on the unproved assumption that the frequency of resistance alleles for an insect control protein will decline when selection pressure for that protein is discontinued.

A mosaic planting of separate fields with hybrids containing different genetic control mechanisms is also an appealing strategy because of its simplicity. However, there is little experimental evidence evaluating its utility. Also, implementation to provide a true mosaic would depend on farmer selection of hybrids with unique insect control proteins.

2. Refugia strategies

Effective refuges allow survival of susceptible ECB and subsequent intermating with individuals that may have been selected for resistance. There is nearly universal agreement that refugia offer an excellent possibility to delay or prevent ECB resistance. However, there is also a critical lack of information about the relative merits of various refugia strategies (Tabashnik, 1994). McGaughey (personal communication) warns that "the refuge must be scaled in time or space such that movement of *feeding* insects is minimized (so that survival is at a minimum), yet the scale cannot be so big as to lose the desired effects of the movement of *mating* insects." Establishing and maintaining this balance between localized feeding control and random, area-wide mating for ECB obviously depends on the particular interaction of ECB, corn cultural conditions and the 176 ICP.

Seed mix

A mix of some percentage of resistant and susceptible plants in one field is one of the most obvious refugia strategies. This option offers the advantage of a clear mix of selected and unselected ECB for mating. However, if feeding ECB can move between resistant and susceptible plants, the net effect of a seed mixture could be to reduce the overall dose to which ECB are exposed. This could enhance the development of ECB resistance (McGaughey, personal communication). There is evidence that neonate ECB larvae migrate from 176 ICP plants at a higher rate than control plants (Gould, personal communication). Although these results are preliminary, they could indicate a disadvantage of seed mixture refugia for transgenic corn and ECB.

Field borders, strips

ECB moths leave corn fields during the day to rest and mate in nearby grassy areas. Field borders or strips of susceptible corn could allow susceptible ECB to survive and mate with surviving ECB from 176 ICP plants. However, the success of this strategy relies on the voluntary cooperation of individual growers to plant areas of susceptible corn, resulting in short-term damage to part of their crop for the long-term benefit of resistance management.

Adjacent fields

Because of the flight and mating behavior of ECB, adjacent fields of susceptible corn could serve as refugia for 176 ICP fields. The success of this strategy is dependent on incomplete adoption of 176 ICP corn by growers for protection from ECB. During at least the first several years following commercialization of 176 ICP corn, it is likely that such a mosaic of susceptible and resistant corn will provide adequate refugia for susceptible ECB.

Time

Rotation to other crops or susceptible corn could create a refuge in time for susceptible ECB. However, given the moderate flight ability of ECB and the predominance of corn in the midwestern U.S., this strategy is not feasible. It is unlikely that enough growers would agree to cooperate in the large area required for the success of such a strategy.

Ciba Seeds' Resistance Management Activities

As described in this document, the immediate risk of ECB resistance to 176 ICP is not significant. Nevertheless, Ciba Seeds is committed to the development of effective strategies to reduce this risk. These activities must account for ECB behavior and genetics, agricultural practices and trends, and the specific expression and efficacy of the 176 ICP. Models and experience with other crops or insects may not be fully applicable for this situation. Much remains to be learned about the behavior and especially the genetics of ECB in relation to the probability of resistance to 176 ICP. There is considerable debate about the merits of potential actions. As indicated above, it is possible that some options for resistance management strategies could actually be counterproductive and enhance the probability of resistance. Ciba Seeds' resistance management strategy for hybrids with 176 ICP will necessarily evolve from the activities outlined below as additional knowledge is obtained through research and observations in commercial fields.

Monitor ECB Susceptibility

Projects to monitor ECB susceptibility levels are essential to obtain genetic information about resistance and to effectively manage 176 ICP products. Ciba, in conjunction with two other industry partners, is supporting a monitoring project conducted by Dr. Blair Siegfried at the University of Nebraska. Initial studies have established a standardized assay and monitored baseline susceptibility levels of ECB populations in Nebraska. Future work will include an assessment of baseline susceptibilities for all major corn growing regions. As corn with 176 ICP or other CryIA(b) insect control proteins enters the market, ECB resistance levels will be monitored in areas with a high concentration of hybrids containing 176 ICP. Ciba Seeds will continue to support this type of work.

Develop and Deploy New Genetic ECB Control Products

Gene products with different modes of action can be deployed to reduce the probability of resistance to 176 ICP. If additional ECB control products are commercialized, ECB exposure to any one insect control protein will be reduced. The probability of resistance to any one product will also be reduced. Ciba has an active research program for the development of new options for insect control. Alternative genes have been identified and are being developed.

Conduct and Support Research on ECB Resistance

The need for additional information on ECB resistance topics has been noted throughout this document. Ciba has conducted and supported such research.

Research projects will continue to be supported with financial and in-kind assistance in each of three critical areas:

- ECB resistance genetics: Numerous genetic issues relevant to ECB resistance to 176 ICP are unresolved. Ciba has supported laboratory selection studies for resistant ECB by Dr. Cliff Keil, University of Delaware, and Dr. Guy Riba, Institut National de la Recherche Agronomique (France) in order to better understand the potential for ECB resistance. Field-based studies concerning the effects of 176 ICP selection pressure and gene flow are also needed in order to better manage potential ECB resistance.

- Refugia: Ciba has supported an on-going research project with Dr. Fred Gould, North Carolina State University, to investigate the potential effects of mixtures of plants with and without 176 ICP on the development of ECB resistance. Additional research should be conducted to compare the efficacy of seed mixtures with the refugia available in adjacent fields.

- Gene deployment: Appropriate deployment strategies for multiple genes need to be determined. Modeling studies have been reported, but work is needed that specifically addresses the corn-ECB complex. In particular, the relative merits of pyramiding genes and temporal deployment of genes need to be assessed.

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Appendix 1

Respondents to Ciba Seeds' Resistance Management Questionnaire

Dr. Richard ffrench-Constant

University of Wisconsin-Madison
Department of Entomology
237 Russell Laboratories
1630 Linden Drive
Madison, Wisconsin 53706

Dr. William H. McGaughey

United States Department of Agriculture - Agricultural Research Service
Northern Plains Area U.S. Grain Marketing Laboratory
1515 College Avenue
Manhattan, Kansas 66502

Dr. David Onstad

University of Illinois at Urbana-Champaign
Agricultural Entomology
172 Natural Resources Building
607 East Peabody Drive
Champaign, Illinois 61820

Dr. Bruce Tabashnik

University of Hawaii
Department of Entomology
3050 Maile Way, Room 310
Honolulu, Hawaii 96822

Dr. John Witkowski

Department of Entomology
University of Nebraska
Northeast Research and Extension Center
Concord, Nebraska 68728

Appendix 2

Ciba Seeds Resistance Management Questionnaire

ASSESSMENT OF RISK

ECB RESISTANCE TO Bt CORN

Ciba Seeds

June 6, 1994

1. Assumptions

A. Ciba Seeds and Mycogen Plant Sciences will be the first companies to sell Bt corn. Each company will sell corn containing Ciba's 176 event (CryIA(b)). The combined market share of these two companies is approximately 5%. Other seed companies, representing over 50% market share, will begin selling Bt corn one year after Ciba and MPS. All companies will sell ECB resistant corn which contains a CryIA(b) protein.

B. Initial sales of Bt corn will be focused in the western corn belt, primarily Nebraska. However, within three years of the first introduction, Bt corn will be readily available throughout the corn belt.

C. Bt corn hybrids will have a 75% market share in the western corn belt within five years of the first introduction. Bt corn market share in other parts of the corn belt will increase to 50% within ten years after the first introduction.

D. No first brood ECB larvae survive on Ciba event 176 plants.

E. Silks of Ciba Bt event 176 plants contain little or no Bt protein. A small percentage of second brood ECB larvae have been observed to survive in the silks, migrate to the lower stalk, bore into the stalk and survive into the winter. Sublethal selection for tolerance to Bt may occur among these individuals.

Assume that 1 in 1000 Bt plants will contain a live second brood ECB larva which survives after feeding on silk or stalk tissues and reproduces the following year.

F. Corn hybrids may legally contain up to 5% off-type plants due to impurities in parent stock or contamination from blow-in pollen during hybrid production. Depending on which hybrid parent is transgenic, these plants may not contain the Bt gene. Assume that 1 in 1000 plants in Bt corn fields will not contain Bt and will be infested by ECB larvae which survive to reproduce.

2. ECB Biology

A. Is ECB utilizing alternate hosts in the midwest? What are they, where are they located by region and in relation to corn fields, and how widespread are they?

B. What is known about mating behavior of ECB, especially between those individuals from corn and those from alternate hosts? What is the likelihood of mating between ECB from corn and from alternate hosts?

C. How far do corn borers fly before mating? How far will they fly before laying eggs after mating?

D. Are there distinct subspecies populations of ECB? If so, do any mating barriers exist between them? If there are mating barriers, how are the populations distributed geographically?

3. Risk Assessment

A. Under what conditions could the use of Bt microbial products hasten ECB resistance if they contained one component the same as transgenic corn but other components with different modes of action?

B. Under what conditions would a second transgenic crop with the same active ingredient as transgenic corn, e.g. CryIA(b) Bt endotoxin, hasten development of ECB resistance?

C. If no resistance management practices are implemented and all ECB-resistant corn expresses only CryIA(b) in high doses, what is your assessment of the extent of ECB resistance to Bt corn as a function of years after introduction? Where will resistance first appear? How fast could resistant genotypes spread to other areas?

D. Describe the hazard presented by ECB resistant to CryIA(b)-expressing corn. What would be lost to the corn grower? to the consumer? to growers of other crops? to society in general?

4. ECB Resistance Genetics

A. What is your estimate of the frequency of a resistance allele in the ECB population which will allow survival on Ciba event 176 Bt plants?

B. Why is there no ECB resistance to current pesticides?

C. What kinds of ECB resistance mechanisms can be envisaged that would overcome Ciba's event 176? What is the probability that these resistance mechanisms would result in lower insect fitness in the absence of event 176 selection pressure?

5. ECB Resistance Management - Refugia

Non-transgenic corn, non-Bt expressing corn tissue, or other host plants may act as a host for susceptible ECB. It has been proposed that these plants or tissues may act as a refuge to maintain susceptible ECB in a predominately CryIA(b) corn culture. At least six scenarios are possible to utilize refugia in a resistance management strategy:

- Tissue specific expression which allows a percentage of ECB to survive and reproduce on transgenic plants.
- Susceptible corn intermixed with resistant corn in the same field.
- Susceptible corn planted in borders or strips next to resistant corn.
- Susceptible corn planted in fields within one mile of resistant corn.
- Susceptible corn planted in fields one to five miles from resistant corn.
- Alternate hosts around resistant corn fields.

A. Is the concept of refugia valid?

B. Do you recommend consideration of other refugia strategies?

C. For any refugia strategy, what percentage of susceptible ECB must survive to maintain resistant individuals below 5% of the total population for 5 years? 10 years?

D. Which of the strategies do you consider a viable option for resistance management? For those strategies which are useable, what type of susceptible corn tissue or percentage of susceptible corn plants is necessary for successful resistance management?

E. Prioritize acceptable strategies by their desirability.

6. Resistance Management - Genetic Strategies

Consider these strategies in terms of expected efficacy for delaying resistance. Assume that all genes are equally efficacious.

- Single gene with a very high dose (10X above LC99).
- Two genes with different modes of action in same plant.
- Two genes with different modes of action in different plants in same field.
- Two genes with different modes of action in different fields in same season.
- Two genes with different modes of action rotated in hybrid products over time.

A. Do you recommend consideration of other strategies?

B. Are there conditions under which the use of any two-gene strategy could result in ECB resistance faster than the single-gene strategy?

C. Which of the strategies, including your own, do you consider a viable option for resistance management? Recommend how you would implement these strategies.

D. Prioritize acceptable strategies by their expected effectiveness in delaying ECB resistance to Bt corn.

7. Resistance Management - General

A. Can you recommend strategies to delay ECB resistance to Bt corn in addition to those which have already been discussed?

B. How can Bt corn be utilized in Integrated Pest Management programs?

REFERENCE DOCUMENT II

ASSESSMENT OF POSSIBLE RESISTANCE TO TRANSGENIC CORN CONTAINING EVENT 176 INSECT CONTROL PROTEIN IN CORN INSECTS OTHER THAN EUROPEAN CORN BORER

submitted by

Ciba Seeds
Ciba-Geigy Corporation
3054 Cornwallis Road
Research Triangle Park, NC 27709-2257

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ASSESSMENT OF POSSIBLE RESISTANCE TO TRANSGENIC CORN CONTAINING EVENT 176 INSECT CONTROL PROTEIN IN CORN INSECTS OTHER THAN EUROPEAN CORN BORER

Stephen M. Sanborn, Ph.D.
Mycogen Plant Sciences

SUMMARY

Assessments of the possibility of resistance to the Bt-based insect control protein (176 ICP) expressed in transgenic corn were made for several lepidopterous corn insects by examining reproductive isolation, reproduction capability, and anticipated exposure to 176 ICP. These factors have been identified as important in the development of resistance in diamondback moth to Bt-based insecticides. Insects assessed were armyworm, black cutworm, corn earworm, common stalk borer, fall armyworm, lesser corn stalk borer, southern corn stalk borer, southwestern corn stalk borer, and western bean cutworm. The possibility that resistance could develop was assessed as low for all but two of these species. The possibilities that 176 ICP resistance could develop in southwestern corn borer and southern corn stalk borer are assessed as medium. Since Bt-based insecticides are neither very effective nor extensively used for the control any of these insects in corn, the risk to corn production should resistance to 176 ICP occur is very low.

INTRODUCTION

CORN CONTAINING EVENT 176 INSECT CONTROL PROTEIN: Mycogen Plant Sciences and Ciba Seeds have developed corn from a transgenic event (named event 176) that expresses a CryIA(b) insect control protein (176 ICP) from *Bacillus thuringiensis* (Bt) subsp. *kurstaki* strain HD1. The gene promoters used in this new corn cause the 176 ICP to be preferentially expressed in pollen and green tissue but minimally expressed in roots, pith, or kernels. The transgenic corn has been shown to effectively reduce yield losses due to damage from the European corn borer (ECB), *Ostrinia nubilalis*. Ciba Seeds has developed a document, *Assessment And Management of European Corn Borer Resistance to Transgenic Corn Containing the Event 176 Insect Control Protein*, which addresses resistance management issues for European corn borer. The purpose of this present document is to assess the possibility of the development of resistance to 176 ICP among lepidopterous corn insect pests other than ECB.

INSECTS ASSESSED: The insecticidal spectrum of 176 ICP is limited to certain lepidopterous species. Therefore, only lepidopterous insect pests of corn of at least minor importance (Metcalf and Luckman, 1982) are discussed below. These are:

Common Name	Scientific Name	Pest Status
Armyworm (true)	*Pseudaletia unpuncta*	Minor to moderate
Black cutworm	*Agrotis ipsilon*	Major but sporadic
Corn earworm	*Helicoverpa zea*	Major and consistent
Common stalk borer	*Papaipema nebris*	Minor to moderate
Fall armyworm	*Spodoptera frugiperda*	Major and consistent

Lesser corn stalk borer	*Elasmopalpus lignosellus*	Minor to moderate
Southern corn stalk borer	*Diatraea crambidoides*	Minor to moderate
Southwestern corn borer	*Diatraea grandiosella*	Major but sporadic
Western bean cutworm	*Loxigrotis albicosta*	Minor to moderate

RISK TO CORN PRODUCTION OF RESISTANCE IN INSECTS OTHER THAN ECB: The risk to corn production is very small should any of the above pests develop resistance to 176 ICP. Should such resistance occur, it would pose little or no hazard to the use of Bt-based pesticides, since these products are neither highly effective nor used extensively for the control of any of the above mentioned insects on corn. In addition, the development of resistance to chemical insecticides is often exacerbated by insecticidal removal of important beneficial insects. This would not be the case should resistance develop in any of the above corn pests, since 176 ICP poses no hazard to beneficial insects.

FACTORS FOR ASSESSING POSSIBILITY OF RESISTANCE TO 176 ICP: The development of insecticide resistance in insect populations results from an evolutionary interplay of insect genetic factors and environmental factors. There have been numerous reports of insect resistance to chemical insecticides, nearly from the beginning of their widespread use in agriculture. However, the development in the field of resistance to Bt-based insecticides during 30 years of use is a very recent phenomenon, exemplified mainly by diamondback moth (*Plutella xylostella*) resistance to Bt in Hawaii, continental US, and Asia (Shelton, et al. 1993, Tabashnik, et al. 1990, Talekar, 1992). The examples of diamondback moth populations that have developed resistance to Bt-based insecticides provide insight into important factors to consider when assessing the possibility of other insects developing resistance to 176 ICP.

Three important factors in the development of resistance to Bt in diamondback (DBM) moth have been identified by Koziel, et al. (1993). First, resistance developed in reproductively isolated DBM populations that had little immigration. Reproductive isolation was, in part, a result of DBM's weak flight ability and its utilization of only cabbage and related species of plant hosts. Thus, resistant DBM were likely to mate with each other and increase the frequency of the resistance genes in the population. Second, DBM has a high reproduction capability and produces many generations per year (as often as every two weeks). This further increased the possibility of resistant individuals mating and made possible rapid increases in the number of resistant progeny in the population. Third, DBM populations were exposed continuously to Bt-based insecticides which favored survival of resistant individuals. These three factors (reproductive isolation, reproduction capability, and Bt exposure) are examined below for each of the lepidopterous pests of corn under consideration.

ASSESSMENT OF THE POSSIBILITY OF 176 ICP RESISTANCE

There is a low possibility of resistance to 176 ICP expressed in transgenic corn for most of the lepidopterous pests of corn. The three factors identified above, reproductive isolation, reproduction capability, and exposure, were used to assess the possibility of resistance to

176 ICP developing among major and minor lepidopterous pests of corn (excluding European corn borer). Each insect species was assessed relative to the diamondback moth example. Assessments of the possibility for resistance to 176 ICP are summarized in Table 1 and discussed in detail in the sections that follow.

REPRODUCTIVE ISOLATION: For most insects, the possibility for reproductive isolation was assessed to be low in comparison with the diamondback moth example. Two considerations are important. First, most of the insects can survive on a wide range of host plants other than corn. Often, these non-corn hosts are plentiful in the corn growing regions and will provide food sources in which susceptible insects can survive. Second, these insects are all strong fliers and can disperse considerable distances to mate or oviposit. This ability means that if there are any resistant survivors, there is a high possibility that they could emigrate from a transgenic corn field to mate with more numerous susceptible individuals from conventional corn or alternate hosts (or vice versa).

The possibilities for reproductive isolation in southwestern corn borer and southern corn stalk borer were assessed as medium relative to diamondback moth. Both species utilize corn as their principal host but also will survive on sorghum, johnsongrass and related grasses. However, each of these species disperses well, which will somewhat offset the effects of relatively narrow host ranges.

REPRODUCTIVE CAPABILITY: For each insect, the reproductive capability was assessed to be low in comparison with the diamondback moth example. In the areas where DBM developed resistance to Bt, this insect often completes 20 generations/year. Thus, a resistant survivor which finds a mate can rapidly increase its numbers. In contrast, in the midwestern US, the insects which were assessed have a minimum of one generation per year (western bean cutworm, common stalk borer) to a maximum of three generations per year (armyworms). In the southern US, reproductive capability of some insects is slightly greater than in the midwest (e.g., four generations per year for southern corn stalk borer), but it is still quite low in comparison with that for DBM.

EXPOSURE TO 176 ICP: For insects that feed on leaves or pollen, the degree of exposure to 176 ICP while feeding in transgenic corn is anticipated to be high, since the 176 ICP will be present in plant tissue constantly and at high levels, compared with foliar application of traditional Bt-based insecticides. Two insects, corn earworm and common stalk borer, are anticipated to have low exposure because they feed primarily on tissues in which 176 ICP is minimally expressed.

Table 1. Assessments of the possibility of resistance to 176 ICP among corn insect pests other than European corn borer.

	Assessment Factors			
Insect	Reprod. Isolation*	Reprod. Capability*	176 ICP Exposure	Possibility of Resistance
Armyworm	Low	Low	High	Low
Black Cutworm	Low	Low	Med	Low
Corn Earworm	Low	Low	Low	Low
Common Stalk Borer	Low	Low	Low	Low
Fall Armyworm	Low	Low	High	Low
Lesser Corn Stalk Borer	Low	Low	High	Low
Southern Corn Stalk Borer	Med	Low	High	Med
Southwestern Corn Borer	Med	Low	High	Med
Western Bean Cutworm	Low	Low	Med	Low

*Low, Medium or High relative to the example of Bt-resistant diamondback moth on cole crops.

ASSESSMENTS BY SPECIES

Information on the host ranges and biology for each species was gathered from standard entomology texts (Metcalf, et al. 1962, Davidson and Lyon, 1987) as well as in personal communications with university personnel. The level of susceptibility to CryIA(b) is not known for some of the insects reviewed in this document. Therefore, information on the susceptibility of these insects to Bt-based insecticides which contain CryIA(b) was gathered from the results of field efficacy evaluations on various crops conducted by Ciba, Mycogen, and university personnel. These products contain a mixture of Bt proteins which may have different binding sites in the insect. Therefore, this information is only an estimate of the probability for CryIA(b) susceptibility in a particular species.

Armyworm, *Pseudaletia unpuncta*

CORN PEST STATUS: Moderate to minor. Armyworms occur in most of the US east of the Rocky Mountains.

Bt SUSCEPTIBILITY & EXPOSURE: Armyworms are moderately susceptible to, but rarely treated with, Bt-based pesticides on any of the crops they infest. Since armyworms feed on leaves, exposure to 176 ICP will be high.

REPRODUCTIVE ISOLATION: Strong flying and dispersal ability of armyworms, coupled with their utilization of numerous host plants (including wheat, corn, oats, barley, rye and many grass species) indicate a low degree of reproductive isolation for armyworms relative to the diamondback moth example.

REPRODUCTION CAPABILITY: Armyworms are widely distributed and typically have three generations of larvae per year in the northern states, but they seldom infest the same crop for more than a single generation. Thus, the capability of a resistant armyworm genotype to rapidly increase its numbers is low, relative to diamondback moth (20 generations/year).

POSSIBILITY OF RESISTANCE TO 176 ICP: LOW. It is unlikely that armyworms could develop resistance to 176 ICP. This is because large numbers of 176 ICP-susceptible armyworms will live on conventional corn and many non-corn host plants and rapidly dilute the effect on the population as a whole from any 176 ICP-resistant armyworm that might survive on transgenic corn.

Black Cutworm, *Agrotis ipsilon*

CORN PEST STATUS: Major but sporadic. Black cutworm is widely distributed throughout the corn production areas of the US.

Bt SUSCEPTIBILITY & EXPOSURE: Black cutworm, and cutworms in general, are not susceptible to CryIA(b). Cutworms are rarely, if ever, exposed to Bt insecticide applications in any of the many crops they infest. Exposure to 176 ICP while feeding on leaves will be high and exposure during stalk or underground feeding will be low. Therefore, the average exposure of black cutworm to 176 ICP is likely to be medium.

REPRODUCTIVE ISOLATION: Black cutworm infests many cultivated crops including vegetables, corn, cotton, tobacco, and sunflower. Many weed species, particularly grasses, are also utilized. The utilization of numerous hosts and its strong dispersal abilities indicate a low possibility for reproductive isolation for black cutworm in comparison with diamondback moth.

REPRODUCTION CAPABILITY: Black cutworm is widely distributed and has two generations per year in northern states, including the midwest corn belt, and about four per year in the south. Relative to diamondback moth, black cutworm has a low capability for rapidly increasing its numbers.

POSSIBILITY OF RESISTANCE TO 176 ICP: LOW. Because black cutworm is not susceptible to Bt, it is unlikely that this insect will be affected by 176 ICP.

Corn Earworm, *Helicoverpa zea*

CORN PEST STATUS: Major and consistent. Corn earworm is found nearly everywhere corn is grown in the US although it can be rare in northern production areas.

Bt SUSCEPTIBILITY & EXPOSURE: Corn earworm (CEW) larvae are very susceptible to the CryIA(b) protein . However, Bt-based insecticides are not effective for control of CEW in corn because of the insect's behavior. Eggs are laid in the silks and newly hatched larvae crawl down the silk channel to the kernels where they begin to feed. Because Bt insecticides must be ingested for control and sprays cannot cover inside the ear, CEW larvae, although susceptible, are not exposed to Bt in corn. Transgenic corn from event 176 has minimal expression of the Bt protein in the kernels. Therefore, it is anticipated that exposure of CEW to 176 ICP will be very low. On the rare occasions when CEW infests whorl stage corn and feeds on leaves, exposure to 176 ICP would be high.

REPRODUCTIVE ISOLATION: Corn earworm overwinters no further north than 39 degrees N latitude (e.g., Kansas) but is a strong flier and migrates to infest corn as far north as Minnesota. This insect survives on corn, cotton, tomato, beans, alfalfa, clover vetch, tobacco, pepper, lettuce, peanuts, and numerous weed hosts. Therefore, the possibility of reproductive isolation in CEW is low compared with diamondback moth.

REPRODUCTION CAPABILITY: Corn earworm has a single generation per year of larvae in most of the midwestern corn belt and as many as six per year in the extreme southern US. Because CEW must migrate each year to infest most of the corn acres in the midwest, the risk of 176 ICP resistance developing due to its reproduction capability is low.

POSSIBILITY OF RESISTANCE TO 176 ICP: LOW. It is unlikely that CEW could develop resistance to 176 ICP. This is because exposure of CEW larvae to 176 ICP while feeding on transgenic corn is expected to be very low. In addition, the conventional corn and multiple non-corn hosts infested by this insect will produce a constant supply of 176 ICP-susceptible CEW that will dilute the effect on the population as a whole from any 176 ICP-resistant individuals.

Common Stalk Borer, *Papaipema nebris*

CORN PEST STATUS: Moderate to minor. Common stalk borer is widely distributed east of the Rocky Mountains.

Bt SUSCEPTIBILITY & EXPOSURE: Common stalk borer (CSB) has not been very susceptible to Bt-based insecticides, perhaps because large larvae move from grassy weed hosts to adjacent corn, where they bore immediately into stalks without feeding extensively on leaves. Typically, Bt insecticides are very effective on small, newly hatched larvae that feed on leaves, but they are not effective on large larvae boring inside plants. Occasionally CSB are exposed to Bt insecticides applied for first brood European corn borer control. Exposure of CSB to 176 ICP while feeding on transgenic corn would be low because it feeds by boring inside the corn stalk where the protein is found only at very low levels.

REPRODUCTIVE ISOLATION: The very wide host range for CSB includes corn, cotton, potato, tomato, and many species of grass and broadleaf weeds. This, and CSB's good flight abilities, indicate a low degree of reproductive isolation for this insect relative to diamondback moth.

REPRODUCTION CAPABILITY: Common stalk borer has only one generation per year. Thus, capability for any resistant CSB to rapidly increase their numbers is very low in comparison with diamondback moth.

POSSIBILITY OF RESISTANCE TO 176 ICP: LOW. The low exposure of CSB to 176 ICP, CSB's ability to survive on a wide range of plants, and CSB's single generation per year make it unlikely that this insect could develop resistance to 176 ICP.

Fall Armyworm, *Spodoptera frugiperda*

CORN PEST STATUS: Major and consistent. Fall armyworm is widely distributed in the mid-south and southeastern US. In some seasons it extends into most of the northern US corn production areas.

Bt SUSCEPTIBILITY & EXPOSURE: Fall armyworm (FAW) is not susceptible to CryIA(b). It is occasionally exposed to Bt sprays in cotton and vegetable production areas of the mid-south and southeast US. Bt-based insecticides are not used for FAW control on corn. Exposure of FAW to 176 ICP while feeding on transgenic corn would be high because of its leaf feeding habit.

REPRODUCTIVE ISOLATION: Fall armyworm survives on many cultivated crops including corn, cotton, alfalfa, clover, peanuts, tobacco, and vegetable crops. In addition, FAW utilizes many grass and broadleaf weed species. Fall armyworm only overwinters in areas where the ground does not freeze, but it is a strong flier and migrates each year to infest corn as far north as Minnesota late in the growing season. These factors indicate a low possibility for reproductive isolation for FAW in comparison with diamondback moth.
REPRODUCTION CAPABILITY: There are multiple generations of larvae per year in the extreme southern US, but only one in much of the midwest US corn belt. Therefore, FAW's reproduction capability is quite low relative to that for diamondback moth (20 generations per year).
POSSIBILITY OF RESISTANCE TO 176 ICP: LOW. Because FAW is not susceptible to Bt, it is unlikely that this insect will be affected by 176 ICP.

Lesser Corn Stalk Borer, *Elasmopalpus lignosellus*
CORN PEST STATUS & DISTRIBUTION: Moderate to minor. Lesser corn stalk borer occurs in the mid-south and southeastern corn producing areas of the US.
Bt SUSCEPTIBILITY & EXPOSURE: Little is known about the effectiveness of Bt-based insecticides against LCSB. However, Bt-based insecticides are not used for LCSB in the field. Exposure of LCSB to 176 ICP while feeding on transgenic corn likely would be low, since neonate larvae rapidly tunnel into corn without feeding extensively on leaves.
REPRODUCTIVE ISOLATION: Lesser corn stalk borer infests corn, cowpea, sorghums, wheat, beans, peas, peanuts, turnips, and many species of grasses. The wide host range of LCSB and its strong dispersal capabilities indicate a low degree of reproductive isolation for this insect relative to diamondback moth. In its range, it is considered primarily as a pest of peanuts and is thought of as a non-pest of corn.
REPRODUCTION CAPABILITY: Lesser corn stalk borer has only two generations of larvae per year (versus 20 per year for DBM). Thus, risk of 176 ICP resistance due to LCSB's reproductive capability is low.
POSSIBILITY OF RESISTANCE TO ICP 176: LOW. There is a low possibility that LCSB could develop resistance to 176 ICP. Selection pressure for resistance to 176 ICP in LCSB will be low on transgenic corn. In addition, the effect of any such survivors will be rapidly diluted by 176 ICP-susceptible individuals dispersing from conventional corn and the other host species.

Southern Corn Stalk Borer, *Diatraea crambidoides*
CORN PEST STATUS & DISTRIBUTION: Moderate to minor. Southern corn stalk borer (SCSB) occasionally occurs in corn along the eastern seaboard. Outside this area it occurs only rarely from Maine to Mississippi.
Bt SUSCEPTIBILITY & EXPOSURE: The susceptibility of SCSB to Bt is unknown but, given its similarities to Southwestern corn borer, it's likely to be moderately susceptible (see Southwestern corn borer section below). As with the lesser corn stalk borer, however, Bt-based insecticides are not used for SCSB control in the field. Exposure of SCSB on transgenic corn to 176 ICP on will be high since this insect feeds on leaves prior to boring into stalks.

REPRODUCTIVE ISOLATION: Southern corn stalk borer infests corn, sorghums, and related grasses (e.g., johnsongrass). Compared to the insects considered previously, SCSB utilizes a narrower range of hosts. When this insect occurs in areas where sorghums are not planted, corn will be the principal host. Sorghum-related grasses are generally considered weeds, and there are very effective herbicides for their control. Therefore, these grasses probably will not serve as significant hosts for SCSB. However, SCSB disperses well and this feature of its biology should offset the effect of a narrower host range. Therefore, the possibility of reproductive isolation for SCSB is assessed as medium, relative to the high degree of isolation for diamondback moth.

REPRODUCTION CAPABILITY: In the northern part of this area it has two generations per year, while as many as four per year occur in the southern part of its range. The risk that 176 ICP resistant SCSB could rapidly increase their numbers is low in comparison with DBM.

POSSIBILITY OF RESISTANCE TO ICP 176: MEDIUM. There is a medium possibility that SCSB could develop resistance to 176 ICP. Selection pressure for resistance to 176 ICP in SCSB will be high on transgenic corn, but the effect of any such survivors will be diluted by a low reproductive capability and 176 ICP-susceptible individuals dispersing from conventional corn and other host species.

Southwestern Corn Borer, *Diatraea grandiosella*

CORN PEST STATUS: Major but sporadic. Southwestern corn borer is very similar to southern corn stalk borer but occurs routinely in the high plains of Texas, Oklahoma panhandle and southern Kansas. It occasionally is a problem in Arkansas, the Mississippi delta , the Missouri bootheel, Tennessee and Kentucky. Its northern range is limited by an inability to overwinter where soil freezes consistently during the winter.

Bt SUSCEPTIBILITY & EXPOSURE: Southwestern corn borer (SWCB) is susceptible to CryIA(b). Bt-based insecticides are seldom used for SWCB control in the field. This is probably due to difficulties with timing and little residual efficacy. Exposure to 176 ICP would be high on transgenic corn since SWCB larvae feed on leaves before boring into stalks.

REPRODUCTIVE ISOLATION: Southwestern corn borer survives on corn, sorghums (grain and forage types) and johnsongrass. Like European corn borer, SWCB adults will fly considerable distances to oviposit. While these factors indicate a low degree of reproductive isolation for SWCB in most of its range, isolation could occur in western irrigated corn where alternate hosts are rare.

REPRODUCTION CAPABILITY: There are two to three generations of SWCB larvae per year, indicating a low possibility of 176 ICP resistance due to this insect's reproduction capability.

POSSIBILITY OF RESISTANCE TO ICP 176: MEDIUM. There is a medium possibility that SWCB could develop resistance to 176 ICP. Selection pressure for resistance to 176 ICP in SWCB will be high on transgenic corn, but the effect of any such survivors will be diluted by 176 ICP-susceptible individuals coming from conventional corn, sorghum, and johnsongrass. However, in western arid production areas where corn is grown under irrigation and sorghum is not present, the only source of susceptible survivors will be conventional corn (expected to represent at least 20% market acres at 4 years after

introduction of corn with 176 ICP). In such areas, there is a somewhat greater possibility of SWCB becoming resistant to 176 ICP.

Western Bean Cutworm, *Loxigrotis albicosta*

CORN PEST STATUS: Moderate to minor. Western bean cutworm (WBC) is found in most of the western dry bean production areas, and occurs routinely as a pest of corn only in eastern Colorado and western Nebraska. There are occasional reports of WBC from as far east as Iowa.

Bt SUSCEPTIBILITY & EXPOSURE: Western bean cutworm is moderately susceptible to Bt-based insecticides but, due to difficulties with application timing, they are not used for control in the field. Preliminary data from the University of Nebraska indicates that WBC is not susceptible to 176 ICP (J. Witkowski, personal communication). Since WBC larvae may feed on pollen in leaf axils prior to attacking the ear (Pilcher, personal communication) exposure to 176 ICP could be high on transgenic corn. While feeding in the ears, exposure will be low since 176 ICP is minimally expressed in kernels. Thus, the overall anticipated exposure of WBC to 176 ICP is medium.

REPRODUCTIVE ISOLATION: The western bean cutworm, like most Noctuids, is a strong flier and has been observed to fly 40 miles to oviposit on a host crop. Host crops utilized by WBC are numerous, but include principally corn and dry beans. This insect's strong flight ability and its utilization of multiple crops as hosts indicate a low degree of reproductive isolation relative to the diamondback moth.

REPRODUCTION CAPABILITY: The WBC has only one generation per year, indicating a very low capability (in comparison with DBM) for any resistant genotypes to rapidly increase their numbers.

POSSIBILITY OF RESISTANCE TO ICP 176: LOW. There is a low possibility that WBC could develop resistance to 176 ICP. Selection pressure for resistance to 176 ICP in WBC will be high on transgenic corn, but the effect of any such survivors will be diluted by 176 ICP-susceptible individuals dispersing from conventional corn and the other host species. The development of resistance will be additionally slowed by the fact that WBC has only one generation per year.

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University Personnel Contacted:

Dr. Michael Chippendale	University of Missouri.
Dr. Frank Davis	Mississippi State University
Dr. Armon Keaster	University of Missouri
Dr. Stan Pilcher	Colorado State University
Dr. John Van Duyn	North Carolina State University

SUPPLEMENT I

ENVIRONMENTAL RELEASE REPORTS

A SUPPLEMENT TO CIBA SEEDS' PETITION FOR DETERMINATION OF NONREGULATED STATUS OF CORN GENETICALLY ENGINEERED TO EXPRESS THE CRYIA(b) PROTEIN FROM *BACILLUS THURINGIENSIS* SUBSPECIES *KURSTAKI*

submitted by

Ciba Seeds
Ciba-Geigy Corporation
3054 Cornwallis Road
Research Triangle Park, NC 27709-2257

This Document Does Not Contain Confidential Business Information

Environmental Release Report

Permit Number: 92-042-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: June, 1992

Site of Release: Ciba Seeds, Bloomington, McLean Co., IL

Site Contact: Dr. Moez Meghji

Purpose of Release: Introduction of Bt gene into elite Ciba inbred lines by traditional breeding

Results: Routine selfs, backcrosses and new crosses 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 to ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetles, and leafhoppers. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control rust infection. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines in their susceptibility to disease or in their response to chemical applications.

Herbicide tolerance: Pre- and post-emergent herbicide was applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

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

Flowering dates, plant height (with one exception, noted below), plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle and width. One of the transgenic lines was shorter in stature than its non-transgenic isogenic line do to transplant shock.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were bagged prior to pollen shed. Once tassels began to shed and pollen was collected for pollinations, a new tassel bag was secured within a few seconds. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: November, 1992

Means of Plant Disposition: Plants were mowed, the field disked and plowed.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 12 months; soybeans were planted the following season and the field was observed for volunteer corn plants.

Number of Volunteers Observed and Action Taken: Five (5) volunteer corn plants appeared and were destroyed prior to flowering by cutting with a hoe.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by bagging of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number: 92-140-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: December, 1992

Site of Release: Ciba Seeds, Boynton Beach, Palm Beach County, FL

Site Contact: Dane Canfield

Purpose of Release: Introduction of Bt gene into elite Ciba inbred lines by traditional breeding

Results: Routine selfs, backcrosses and new crosses accomplished, seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were infestations of European cornborer at this site and so observable differences in susceptibility between Bt and non-Bt lines did occur. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control various diseases. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre- and post emergent herbicides were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were bagged prior to pollen shed. Once tassels began to shed and pollen was collected for pollinations, a new tassel bag was secured within a few seconds. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: January, 1993

Means of Plant Disposition: Plants were mowed and disked repeatedly into the field.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 12 months. The field was observed visually for volunteer corn plants.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by bagging of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number: 92-140-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: April, 1993

Site of Release: Ciba Seeds, Boynton Beach, Palm Beach County, FL

Site Contact: Dane Canfield

Purpose of Release: Hybrid seed producion

Results: Seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were infestations of European cornborer at this site and so observable differences in susceptibility between Bt and non-Bt lines did occur, with Bt lines showing resistance. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control various diseases. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre- and post emergent herbicides were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: Corn was open pollinated with a 30-day temporal isolation; or spatial isolation of at least 660 feet from surrounding corn . Ciba breeders observed these means of pollen containment to be extremely effective in restricting out-crossing.

Date of Release Termination: July, 1993

Means of Plant Disposition: Plants were mowed and disked repeatedly into the field.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 3 months. The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: 13 volunteer plants were observed during the observation period and destroyed by discing.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by spatial distance from other corn of 660 feet and/or temporal isolation of a minimum of 30 days to prevent cross fertilization. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number: 92-140-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: December, 1993

Site of Release: Ciba Seeds, Boynton Beach, Palm Beach County, FL

Site Contact: Dane Canfield

Purpose of Release: Line increases, test crosses

Results: Seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were infestations of European cornborer at this site and so observable differences in susceptibility between Bt and non-Bt lines did occur, with Bt lines showing resistance. There were a few off-types among the transgenic lines with regard to a lower level of resistance to ECB and these were destroyed. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control various diseases. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre- and post emergent herbicides were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: Plants were stressed due to heavy rainfall, but there were no observable differences between the transgenic and non-transgenic lines.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: Corn was hand-pollinated; All transgenic plants were tassel bagged prior to pollen shed. Ciba breeders observed this means of pollen containment to be extremely effective in restricting out-crossing to surrounding corn.

Date of Release Termination: on-going

Means of Plant Disposition: Plants will be mowed and disked repeatedly into the field.

Time and Method of Monitoring for Volunteer Plants: The plot will be monitored for 3 months. The field will be observed visually for volunteer corn plants, which will be destroyed when found.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by bagging of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number: 93-014-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: April, 1993

Site of Release: Ciba Seeds, Kaunakakai, Molokai, HI

Site Contact: Dr. Elizabeth Johnson

Purpose of Release: Hybrid test crosses

Results: Seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were no infestations of European cornborer at this site and so no observable differences in susceptibility between lines. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control rust infections, northern and southern corn leaf blights. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre-emergent herbicides (Dual and AAtrex) were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were bagged prior to pollen shed. Once tassels began to shed and pollen was collected for pollinations, a new tassel bag was secured within a few seconds. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: August, 1993

Means of Plant Disposition: Plants were disked into the field.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 70 days; with 3 irrigations during that period. The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by bagging of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May, 1993
Site of Release:	Ciba Seeds, Bloomington, McLean Co., IL
Site Contact:	Dr. Moez Meghji
Purpose of Release:	Introduction of Bt gene into elite Ciba inbred lines by traditional breeding, line increases, gene efficacy trials
Results:	Routine selfs, backcrosses and new crosses accomplished, seed increase, efficacy confirmed

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 to ECB. Plants did not differ in their susceptibility to damage by other insects, such as aphids, corn rootworm beetles, and leafhoppers. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control rust infection. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines.

Herbicide tolerance: Pre- and post-emergent herbicide was applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No differences in stress damage observed between lines in this release.

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

Flowering dates, plant height (with one exception, noted below), plant vigor, silk and anther color, tassel branching, cob color and size, physiological maturity dates, and leaf angle and width. One of the transgenic lines was shorter in stature than its non-transgenic isogenic line do to transplant shock.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were bagged prior to pollen shed. Once tassels began to shed and pollen was collected for pollinations, a new tassel bag was secured within a few seconds. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: November, 1994

Means of Plant Disposition: Plants were mowed, the field disked and plowed.

Time and Method of Monitoring for Volunteer Plants: The plot will be monitored for 12 months; soybeans will be planted the following season and the field observed for volunteer corn plants, which will be destroyed by hoe.

Number of Volunteers Observed and Action Taken: Five (5) volunteer corn plants appeared and were destroyed prior to flowering by cutting with a hoe.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by bagging of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May 21, 1993
Site of Release:	Madison co, IA
Site Contact:	Allen Brush
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility. Both transgenic and non-transgenic lines showed equal susceptibility to comon rust.

Herbicide tolerance: Pre-emergent herbicides (Dual and AAtrex) were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No differences were observed. Both transgenic and non-transgenic lines showed similar response to denitrification.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 10/20/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Stalk sample were removed from the plot for futrther analysis. Remaining plants were shredded and disked into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May 18, 1993
Site of Release:	Seward, NE
Site Contact:	Dr. Louis Reeder
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility.

Herbicide tolerance: Pre-emergent herbicides (Dual and AAtrex) were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 10/18/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Stalk sample were removed from the plot for futrther analysis. Remaining plants were shredded and disked into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May, 1993
Site of Release:	Marion, IA
Site Contact:	Robert Miller
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae as compared to non-transgneic control lines

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility, except that Bt corn plants appeared on visual inspection to have less anthracnose stalk rot.

Herbicide tolerance: No differences observed between transgenic and non-transgenic lines with regard to standard herbicide treatments.

Stress tolerance: No differences were observed.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 10/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Remaining plants were disked into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May 8, 1993
Site of Release:	Shelbyville, Shelby Co., IL
Site Contact:	Barry Lawrence
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae as compared to non-transgneic control lines; no differences observed with regard to other traits

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to general numbers and behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility: common rust was present but there were no differences.

Herbicide tolerance: No differences observed between transgenic and non-transgenic lines with regard to standard herbicide treatments.

Stress tolerance: No differences were observed.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 10/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Remaining plants were chiseled into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May, 1993
Site of Release:	Center Farm, McClean Co., IL
Site Contact:	Moez Meghji
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae as compared to non-transgneic control lines; no differences observed with regard to other traits

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to general numbers and behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility.

Herbicide tolerance: No differences observed between transgenic and non-transgenic lines with regard to standard herbicide treatments.

Stress tolerance: No differences were observed.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 11/15/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Remaining plants were shredded and disked into the field. All grain from the plot was ground and then plowed into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	May, 1993
Site of Release:	Northpoint, McClean Co., IL
Site Contact:	Moez Meghji
Purpose of Release:	Gene Efficacy evaluation for ECB tolerance
Results:	Excellent control of both first and second generation ECB larvae as compared to non-transgneic control lines; no differences observed with regard to other traits

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene. In addition, visual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to general numbers and behavior of beneficial insects present in the plot.

Disease susceptibility: Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility.

Herbicide tolerance: No differences observed between transgenic and non-transgenic lines with regard to standard herbicide treatments.

Stress tolerance: No differences were observed.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: All of the transgenic tassels were removed prior to pollen shed. Plots were hand harvested and gleaned Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: 11/15/93

Means of Plant Disposition: All plots were hand harvested and the field was gleaned. Remaining plants were shredded and disked into the field. All grain from the plot was ground and then plowed into the field.

Time and Method of Monitoring for Volunteer Plants: The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by removal of tassels to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer.

Environmental Release Report

Permit Number: 93-014-01

Permittee: Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]

Date of Release: May, 1993

Site of Release: Ciba Seeds, Kaunakakai, Molokai, HI

Site Contact: Dr. Elizabeth Johnson

Purpose of Release: Hybrid test crosses

Results: Seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were no infestations of European cornborer at this site and so no observable differences in susceptibility between lines. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control rust infections, northern and southern corn leaf blights. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre-emergent herbicides (Dual and AAtrex) were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: Spatial isolation of at least 660 feet from surrounding corn. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: September, 1993

Means of Plant Disposition: Plants were disked into the field.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 85 days; with 3 irrigations during that period. The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by spatial distance from other corn of 660 to prevent pollen dissemination. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Environmental Release Report

Permit Number:	93-014-01
Permittee:	Rich Lotstein, Ciba Biotechnology, 3054 Cornwallis Road, Res. Tri. Park, NC 27709 [(919)541-8534]
Date of Release:	June, 1993
Site of Release:	Ciba Seeds, Kaunakakai, Molokai, HI
Site Contact:	Dr. Elizabeth Johnson
Purpose of Release:	Hybrid test crosses
Results:	Seed produced

Observations:

Insect susceptibility: Transgenic and non-transgenic lines were identical in their susceptibility to damage by insects not targeted by the Bt gene, such as thrips, leafhoppers, and spider mites. There were no infestations of European cornborer at this site and so no observable differences in susceptibility between lines. In addition, casual observations by experienced Ciba entomologists, breeders, and agronomists indicated that there were no differences between transgenic and non-transgenic lines with regard to behavior of beneficial insects, such as pollinators (bees and wasps).

Disease susceptibility: Fungicide was applied to control rust infections, northern and southern corn leaf blights. Ciba plant pathologists and breeders observed no differences between transgenic and non-transgenic lines with regard to disease susceptibility or response to fungicide treatment.

Herbicide tolerance: Pre-emergent herbicides (Dual and AAtrex) were applied; no damage observed by Ciba staff to the transgenic or non-transgenic lines.

Stress tolerance: No stress damage observed to any lines in this release.

Physical Characteristics: There were no observable differences between the transgenic and non-transgenic lines with regard to the following characteristics that are observed routinely in the process 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 and width.

Means and Effectiveness of Containment Measures: Spatial isolation of at least 660 feet from surrounding corn. Ciba breeders observed this means of pollen containment to be extremely effective in limiting pollen dispersal.

Date of Release Termination: September, 1993

Means of Plant Disposition: Plants were disked into the field.

Time and Method of Monitoring for Volunteer Plants: The plot was monitored for 70 days; with 3 irrigations during that period. The field was observed visually for volunteer corn plants.

Number of Volunteers Observed and Action Taken: No volunteer plants were observed during the monitoring period.

Additional Comments:

No other plant species that could cross with corn are present in North America, so transfer of the gene to wild, weedy relatives was impossible. As required, the transgenic plants were isolated by spatial distance from other corn of 660 to prevent pollen dissemination to surrounding corn not part of the permitted field test. As a result, there was minimum chance of outcrossing or seed mixture with other corn plantings not part of the transgenic field release.

There was no evidence of any change in the survival characteristics of the transgenic corn plants as compared to non-transgenic lines. There were no changes in Bt Corn relative to non-Bt Corn in the factors that enhance weediness of a plant species, such as ability to survive various stresses (except ECB damage), seed number, viability and germination rate. Although resistant to the chemical herbicide glufosinate, the Bt Corn lines tested are not altered in their sensitivity to other chemical weed control agents.

The transgenic plants demonstrated excellent resistance to damage by European cornborer. The Bt gene and its associated marker segregated as a typical Mendelian inheritance for a single locus.

Ciba Seeds
Agricultural Biotechnology
Ciba-Geigy Corporation
3054 Cornwallis Road
Research Triangle Park, North Carolina 27709-2257
Telephone 919-541-8683 Telefax 919-541-8585

Jeffrey Stein
Regulatory Affairs Manager

January 6, 1995

Dr. Ved Malik, Biotechnologist
Biotechnology Permits, BBEP, APHIS, USDA
Room 850 Federal Building
6505 Belcrest Road
Hyattsville, MD 20782

Dear Dr. Malik,

In November 1994, Ciba Seeds submitted to USDA/APHIS/BBEP a petition for determination of nonregulated status of Ciba Seeds' corn genetically engineered to express the CrylA(b) protein from *Bacillus thuringiensis* subspecies *kurstaki*. This letter is in regards to how Ciba Seeds wishes to have the aforementioned corn designated in the Federal Register. We believe the designation "Event 176 Corn" is appropriate for this purpose. While we may now refer to the previously submitted petition as a petition for deregulation of Event 176 Corn, as defined on page 6 of the petition, we are indeed petitioning for deregulation of Event 176 Corn and all progeny of Event 176 Corn used for feed and food grade field corn, developed and produced by Ciba Seeds as well as industry collaborators of Ciba Seeds, who are incorporating the desired traits into their own proprietary lines of corn for use in feed and foods such as sweet corn, popcorn, and other traditional corn products.

Please do not hesitate to contact me if you have any further questions.

Sincerely,

Jeffrey Stein
Regulatory Affairs Manager

SUPPLEMENT III

THE EFFECT OF BT MAIZE LEAF PROTEIN ON THE SURVIVAL AND REPRODUCTION OF COLLEMBOLA (*FOLSOMIA CANDIDA*)

A SUPPLEMENT TO CIBA SEEDS PETITION FOR DETERMINATION OF NONREGULATED STATUS OF CORN GENETICALLY ENGINEERED TO EXPRESS THE CRYIA(B) PROTEIN FROM BACILLUS THURINGIENSIS SUBSPECIES KURSTAKI

SUBMITTED BY

CIBA SEEDS
CIBA GEIGY CORPORATION
3054 CORNWALLIS ROAD
RESEARCH TRIANGLE PARK, NC 27709-2257

THIS DOCUMENT DOES NOT CONTAIN CONFIDENTIAL BUSINESS INFORMATION

THE EFFECT OF *BT* MAIZE LEAF PROTEIN ON THE SURVIVAL AND REPRODUCTION OF COLLEMBOLA (*FOLSOMIA CANDIDA*)

SUMMARY

A study was conducted to determine the effect of a CryIA(b)-enriched *Bt* maize leaf protein preparation on the survival and reproduction of the collembolan *Folsomia candida*, a soil arthropod. Groups of collembolans were incubated in soil that had been supplemented with a range of concentrations of a leaf protein preparation derived from event 176 maize plants. Both mean adult survival and mean number of offspring declined in the two highest *Bt* maize protein treatment groups, while no statistically significant effect was discerned at a lower concentration. This concentration, where no observable effect on *F. candida* was observed, ranges from >210-fold greater than the estimated exposure level expected under normal agricultural conditions, to 9.5-fold greater than the exposure level that would be encountered under a highly unlikely, worst-case agricultural scenario. We conclude from this study that there should be no adverse effect on the survival and reproduction of *F. candida* as a result of exposure to event 176 *Bt* maize plants.

SURVIVAL AND REPRODUCTION IN *FOLSOMIA CANDIDA*

A 28-day toxicity and reproduction study, employing an OECD draft protocol, was conducted by Springborn Laboratories, Inc. (Wareham, Mass), using the collembolan, *Folsomia candida*. Collembolans were exposed to one of three soil concentrations of protein extracted from *Bt* maize (corn) leaves ("*Bt* maize leaf protein"), protein extracted from nontransgenic maize leaves ("control maize leaf protein"), or untreated soil. Each test or control group consisted of forty 10-12 day-old collembola subdivided into four replicates of 10 animals each. The concentrations of *Bt* maize leaf protein used were 125 mg protein/kg soil, 250 mg protein/kg/soil, and 500 mg protein/kg soil. The CryIA(b) delta-endotoxin content of the *Bt* maize leaf protein was 0.07%, therefore the test concentrations in terms of CryIA(b) were 0.088, 0.175, and 0.35 mg/kg soil. A concentration of 500 mg control maize leaf protein/kg soil was used as a control for possible effects from maize protein *per se*. The collembolans in each replicate of each treatment group were provided with 2 mg yeast as food on days 0 and 14. Since collembolans live within soil substrate, it was not possible to observe the condition of the animals until study termination without unduly disrupting the test system. Adult survival and the number of offspring produced were analyzed at study termination.

Mean adult survival was 100% in the untreated controls, 100% in the nontransgenic maize protein controls, and was 95%, 38%, and 13% in the 125, 250, and 500 ppm transgenic protein groups, respectively. These results indicate that the 28-day LC_{50} was approximately 240 mg protein/kg soil (0.17 mg CryIA(b)/kg). Mean number of offspring in the untreated controls was 147/replicate (range of 113 to 186 per replicate). The mean number of offspring in the control maize protein group was 155/replicate (range of 98 to 260 per replicate). Mean number of offspring/replicate in the 125 ppm, 250 ppm, and 500 ppm *Bt* maize protein groups was 96 (range of 76 to 110), 27 (range of 15 to 43), and 4 (range of 2 to 6), respectively. Adjusted for adult survival, the mean values are 14.7 offspring/adult for the untreated controls,

15.5 offspring/adult for the maize protein controls, and 10.1, 6.9, and 3.3 offspring/adult for the 125, 250, and 500 ppm *Bt* maize protein groups, respectively. Compared to the two control groups, there was a significant decline in the number of offspring produced in the two higher *Bt* maize protein groups, while the offspring population in the 125 ppm group was not significantly different from the controls; therefore, this concentration represents the NOEC. Based on these results, the maximum acceptable toxicant concentration (MATC) range is 125 mg protein/kg soil [0.088 ppm CryIA(b)] to 250 mg protein/kg soil [0.175 ppm CryIA(b)], with a point estimate (geometric mean of the NOEC and LOEC) of 180 mg protein/kg soil [0.126 ppm CryIA(b)].

Very few reports of studies examining the effects of *B.t.* delta-endotoxins on soil invertebrates are available in the literature (reviewed by Addison, 1993). However, the observed sensitivity of *Folsomia candida* to the *Bt* maize leaf protein preparation is consistent with a report of sensitivity of collembolans towards a commercially prepared formulation of *Bacillus thuringiensis* subsp. *galleriae* (*Btg*) (Atlavinyté *et al.*, 1982). While that report suggests that collembolans are sensitive to *Bt* endotoxins, the presence of *Bt* beta-exotoxin, a relatively non-selective toxin, in the tested formulation cannot be ruled out. Sensitivity to other components of the formulation also cannot be ruled out. There are several similarities between *Btg* and *Bacillus thuringiensis* subsp. *kurstaki* (*Btk*), the native source of the CryIA(b) protein which Ciba Seeds' synthetic gene in maize was designed to encode in truncated form. These similarities suggest that, if collembolans are indeed sensitive to *Btg* delta-endotoxins, sensitivity to *Btk* delta-endotoxins is not unexpected. *Btg* produces a CryIG delta-endotoxin, in the same general class as the CryIA(b) protein in *Bt* maize (Smulevitch et al., 1991). *Btg* and *Btk* share a high degree of DNA relatedness (>90% homology) (Nakamura, 1994). In addition, monoclonal antibodies produced against *Btk* crystal protein strongly cross-react to crystal protein derived from *Btg*, indicating that the crystal protein preparations from these two subspecies are very similar (Huber-Lukac *et al.*, 1986). The data suggest that the observed sensitivity of *F. candida* to the *Bt* maize protein preparation is due to the inherent sensitivity of this organism to one or more *Bt* delta-endotoxins

EXPOSURE ESTIMATES

Based on typical agricultural planting practices for corn (number of plants per acre), the average mass of corn plants at various stages of growth, and assays of corn plants at various stages of growth for CryIA(b) content, Ciba Seeds has estimated the quantity of CryIA(b) protein likely to be available for incorporation into soil under normal agricultural practices. These estimates are based on whole plant assays for CryIA(b), the mean weight of the plants assayed, and a value of 25,000 plants per acre. Estimates range from <0.19 g CryIA(b)/acre for senescent (post-harvest) whole plants to a maximum of 4.2 g CryIA(b)/acre for fully-grown plants (maximum vegetative biomass) at anthesis (i.e., before harvest) (see Chapter 4, Table 6 of Petition). The values for senescent plants were all below the assay limit of quantification and the estimate used represents the highest limit of quantification determined for the assay when whole plants were analyzed for CryIA(b) content. Experimentally, Ciba Seeds has reliably achieved an 85% extraction efficiency for CryIA(b) from plant tissue, but a conservative extraction efficiency value of 50% will be used to estimate potential exposure. Thus, the estimates for CryIA(b) become <0.38 to 8.4 g/acre. The former estimate (<0.38 g/acre) represents a more likely exposure scenario because this value is representative of the CryIA(b) content remaining in the plant after harvest, when it is most probable that plants will be tilled into and mixed with the soil. The latter value (8.4 g/acre) is considered extremely

unlikely to occur, as this would be representative of corn plants at maximum vegetative growth (at anthesis) being tilled into the soil, which represents a highly unlikely occurrence. A 6-inch depth was selected as being representative of tillage depths under normal agricultural practices. Using a standard value of 9.08×10^5 kg soil/acre based on a 6-inch soil depth, these quantities of CryIA(b) represent $< 4.19 \times 10^{-4}$ to 9.25×10^{-3} mg CryIA(b)/kg soil. Shallower tillage depths would result in higher estimated CryIA(b) concentrations while deeper tillage depths would result in lower estimated CryIA(b) concentrations. Exposure is further reduced or practically eliminated in fields on which farmers practice reduced tillage or no-till techniques.

Environmental Fate of CryIA(b)

Although specific data on the soil persistence of Ciba Seed's *Bt* corn CryIA(b) are not available, information in the literature suggests that Cry endotoxin proteins are short-lived in the environment. Data generated on a CryIA(c) protein indicate that the half-life of native protein in soil is approximately 12-16 days (Palm et al., 1994). Similar data on the same CryIA(c) protein when remaining intact within plant tissue indicated a half-life of only 4-7 days (Palm et al., 1994). These authors indicate that purified *Bt* delta-endotoxin appears to be more stable than the same toxin incorporated into plant tissue, either because the plant tissue stimulates microbial growth while purified endotoxin does not or because plant degradative enzymes are released during plant tissue decay. Such short half-lives are consistent with the readily digestible proteinaceous nature of Cry delta-endotoxins. This, coupled with an anticipated use pattern of *Bt* maize that primarily consists of a single planting per season should alleviate concerns about a possible build-up of Cry delta-endotoxin proteins to concentrations that might exceed acceptable safety factors.

Hazard Potential

Safety factors based on the collembola study NOEC of 0.088 mg CryIA(b)/kg soil and the estimated soil concentrations range from 9.5-fold for the unlikely scenario of tilling fully green, anthesis-stage plants into the soil to >210-fold for the more likely scenario based on tilling under senescent post-harvest plants. Similar calculations using the point estimate MATC of 180 mg protein/kg soil (0.126 mg CryIA(b)/kg soil) result in safety factors ranging from 13.6-fold for the unlikely pre-harvest scenario to >300-fold for the more likely post-harvest exposure scenario. Thus, it is highly unlikely that collembolans in the field will be impacted under normal agricultural scenarios. Considering the conservative estimate of extraction efficiency used to estimate CryIA(b) content in plants, it is also not anticipated that CryIA(b) will significantly impact collembolans in the unlikely circumstance of a pre-harvest crop being tilled into the soil. An additional consideration is that collembolans prefer to feed on saprophytic fungi found on decaying plant matter, rather than plant matter *per se* (Klironomos et al, 1992). Therefore, collembolans would not be likely to ingest significant quantities of fresh corn plant tissue but may ingest partially digested plant tissue that has been colonized by fungi. It is also likely that the fungi would have degraded some of the CryIA(b) present in the plant tissue.

Data on the Soil Toxicity of Other Corn Insecticides

Very little data are available on the acute or chronic toxicity to collembola of other, conventional insecticides used to control the corn borer. However, available data on the

toxicity to collembolans (*Folsomia candida*) exposed to soil treated with conventional insecticides for 24 hours indicates high mortality of collembola exposed to soil concentrations ranging from 0.05 ppm to 0.5 ppm. Four organophosphorus insecticides commonly used for corn rootworm control--fonofos, chlorpyrifos, phorate, and disulfoton--were among the insecticides tested. Results indicate 100% mortality with a 24-hour exposure to 0.05 ppm fonofos, phorate, or chlorpyrifos, 55% mortality with exposure to 0.05 ppm disulfoton, and 100% mortality with exposure to 0.1 ppm disulfoton (Thompson and Gore, 1972). Therefore, these products appear to present a greater hazard to collembolans on an acute basis than CryIA(b) does with a longer exposure period.

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