Field Evaluation of Transgenic Corn Containing a *Bacillus thuringiensis* Berliner Insecticidal Protein Gene Against *Helicoverpa zea* (Lepidoptera: Noctuidae)[1]

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ABSTRACT Twelve independently transformed lines of transgenic corn (*Zea mays* L.) expressing the CryIA(b) insecticidal protein from *Bacillus thuringiensis* var. *kurstaki* were field tested to evaluate their resistance to the corn earworm, *Helicoverpa zea* (Boddie). Silks of the primary (=top) ears of transgenic [CryIA(b) positive] and isoline control plants [no CryIA(b) protein] were artificially infested with first-instar *H. zea* larvae and the length of ear penetration was measured after 19 d. Eight of the 12 lines had significantly less ear damage than their respective isoline controls; 3 transgenic lines reduced *H. zea* feeding damage by > 75% and stunted surviving *H. zea* larvae. Concentration of the CryIA(b) protein (µg/g fresh weight) in silks of the transgenic lines, determined using ELISA, ranged from 0.0 to 1.28 µg/g. Within transgenic lines, there was a weak ($P < 0.06$) negative relationship between the concentration of CryIA(b) protein in fresh silks and the length of *H. zea* ear penetration.

KEY WORDS Insecta, CryIA(b) protein, transgenic corn, silk, *Helicoverpa zea*

The development of corn transformation technology has allowed the introduction and expression of genes encoding *Bacillus thuringiensis* Berliner insecticidal proteins into the corn genome (Fromm et al. 1990, Gordon-Kamm et al. 1990, Koziel et al. 1993, Armstrong et al. 1995). While the primary target for transgenic corn is the European corn borer, *Ostrinia nubilalis* (Hübner), other important Lepidoptera such as the corn earworm, *Helicoverpa zea* (Boddie), may be controlled or suppressed. *Helicoverpa zea* is perhaps the most important pest of field corn, *Zea mays* L., in the southeastern United States (Wiseman et al. 1984) and can be a severe problem on sweet corn produced for fresh market and processing. Yield losses from *H. zea* damage to field corn are often significant but difficult to predict because of seasonal variability in *H. zea* population levels.

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In the eastern and southeastern United States, susceptible and tolerant corn produce large numbers of *H. zea* that develop on corn and then move to other vulnerable crops such as cotton, *Gossypium hirsutum* L.; soybean, *Glycine max* (L.) Merr.; and peanut, *Arachis hypogaea* L., where larval feeding damage may cause significant economic losses (Wiseman and Morrison 1981).

Helicoverpa zea adults prefer corn silk for oviposition and newly-hatched larvae typically feed on the silk before entering the ear where they complete larval development on the kernels. Because of the *H. zea*-corn silk association, we studied CryIA(b) levels, using ELISA techniques, in silk from transgenic corn grown under field conditions in two locations. In 1994, several lines of transgenic corn, representing independent transformation events, were artificially infested with *H. zea* to determine potential field efficacy.

Materials and Methods

Corn Lines and CryIA(b) Protein. Corn lines expressing the *Bacillus thuringiensis* var. *kurstaki* CryIA(b) insecticidal protein were produced by microprojectile bombardment of embryogenic "Hi-II" callus (Armstrong et al. 1991) followed by selection and regeneration of stably transformed tissue as described by Fromm et al. (1990). Genes conferring resistance to glyphosate, the active ingredient in Roundup™ herbicide, were used as selectable markers to produce the transformed plants tested in 1994 (Howe et al. 1992). The insect resistance genes used were of the *cryIA(b)* class (Höfte and Whiteley 1990, Feitelson et al. 1992), with coding sequence modifications to increase expression in plants (Perlak et al. 1991). Expression in most cases was driven by an enhanced 35S promoter from cauliflower mosaic virus (Kay et al. 1987), with an intron included between the promoter and *cryIA(b)* coding region to increase expression (Callis et al. 1987).

Field Evaluation. Twelve transgenic corn lines homozygous for the *cryIA(b)* gene and their respective isoline controls (segregating families not expressing CryIA(b) protein) were evaluated in 1994 at Jerseyville, IL. The experimental design was a randomized complete block with four replications for the transgenic corn events. Two replications of each isoline control were grown in randomized plots in a separate block. Transgenic lines and isolines were planted in single row plots, 6.1 m in length and 0.76 m apart. Up to 40 seeds were planted per row and later thinned to a maximum of 20 plants/row. Crop production practices common to the midwestern corn belt were used for cultivation. Weeds were controlled post-emergence by hand. The soil type was muscatine.

Helicoverpa zea eggs were obtained from the USDA-ARS, Southern Field Crop Insect Management Laboratory, Stoneville, MS, and allowed to develop and hatch under ~28°C incubation conditions. Newly-eclosed larvae were lightly misted with distilled water and temporarily stored (up to 48 h) at ~10°C prior to transport to the field in refrigerated ice chests. For plant infestation, first-instar larvae were applied directly to silks using a camel's hair brush moistened with tap water.

Ears on plants from all corn lines were infested with *H. zea* at the full silk stage of maturity on 22 July and repeated (same plants and ears) on 23 July

1994. Each day, 15 to 20 *H. zea* larvae were applied to a total of 5 to 6 plants per corn line, in each plot. Larvae were placed in or around the silk channel of the primary (top) ears. Ear damage ratings (total cm of feeding damage measured from the tip of the ear down toward the ear shank) were taken 19 d (11 August) following the second *H. zea* larval infestation.

Helicoverpa zea damage (cm) to ears of transgenic lines was analyzed using analysis of variance (SAS Institute 1989) with the Tukey-Kramer HSD test used to compare means. Individual transgenic lines were compared with their respective isoline control by assuming that location effects, due to the separation of transgenic lines and isolines, were minimized by the uniform insect infestation. Then, pooled standard deviations and standard errors were calculated for each transgenic-isoline pair comparison. The *t*-statistic, with 4 df, was determined as the difference between the mean damage of the transgenic and isoline control divided by the pooled standard error. The relationship between the extent of ear penetration and CryIA(b) protein concentration in silks (µg/g fresh weight) was examined using correlation analysis.

Silk Samples. Green silks were sampled, at Jerseyville, IL, from the secondary ears of plants whose primary ears had been artificially infested with *H. zea* larvae. Silk samples also were collected from the primary ears of transgenic corn plants (not infested with *H. zea*) grown at Molokai, HI in 1994. Silks were typically sampled at the R1 stage (d 2-4 of silking). Silk samples were immediately frozen on dry ice and stored frozen at -80°C prior to processing and ELISA.

Enzyme Linked Immunosorbent Assay (ELISA). Frozen silks were crushed by hand in the sample storage bag then further blended to a powder, in dry ice, using a Waring Blender. Extraction of protein from the powdered silk tissue was done by adding 100 mg of powdered fresh tissue to 2 mL Tris-Borate Extraction Buffer, then homogenizing the tissue using a Brinkman Polytron for ~1 min at a spead of ~17,500 rpm. Ground samples were centrifuged and the extracts decanted. Total protein determinations were performed on the extracts using the BIO-RAD (Bradford) Protein assay (Bradford 1976). Extracts were tested by a CryIA(b) protein double antibody sandwich quantitative ELISA (Clark et al. 1986). Triplicate wells in Nunc Maxi-Sorp plates (Nunc, Kamstrup, Denmark) were used for two dilutions of each sample. Coating and alkaline phosphatase-conjugated rabbit anti-CryIA(b) stocks were at ≈1.0 mg/mL and were used diluted 1:500 (Ab1) or between 1:750 to 1:1000 (Ab2) in the assay. Buffer blanks and extracts of silks from check plants were used as negative controls. During incubation at 4°C, plates were placed in sealed containers with RH > 50%. ELISA values (absorbances at 405 nm with a 640-660 nm reference) were recorded with an Bio-Rad Model 3550 Micro-titer Plate Reader (Bio-Rad, Richmond, CA). Readings were taken 45 to 60 min after the addition of the enzyme substrate (1.0 mg mL^{-1} p-nitrophenyl phosphate). Finally, CryIA(b) protein values in silk extracts (ng/mL) were converted to µg/g protein per fresh weight of tissue. A paired comparison *t*-test was used to examine the relationship, within transgenic lines, between CryIA(b) protein levels in silks of plants grown in Hawaii (first ears) and Illinois (second ears).

Results

Field Evaluation. All twelve transgenic lines tested had less injury than their respective isoline control (Table 1); for 8 lines, this difference was statistically significant (Student t=test, $P < 0.05$). Control isolines were equally susceptible to *H. zea* feeding with damage ranging from 4.8 to 7.0 cm ($\bar{x}$ ± SEM = 5.7 ± 0.7 cm]. The three most resistant transgenic lines had less than 25% of the ear damage scored in their respective isoline controls; the few surviving *H. zea* larvae found on these 3 lines were stunted (mostly 3rd instars) compared with larvae found on control lines (generally 5th-6th instars - data not shown).

CryIA(b) Protein in Silks. CryIA(b) protein in silk ranged from 1.28 μg/g to 0.00 μg/g (Table 1). Within individual transgenic lines, the CryIA(b) protein concentration in silks sampled from top ears (Hawaii) and second ears (Jerseyville) was not significantly different ($t = 0.83$; df = 10; $P > 0.05$).

CryIA(b) Protein in Silks and *H. zea* Damage. CryIA(b) protein concentrations in transgenic silks and the length of *H. zea* ear penetration were negatively correlated. The correlation approached significance when silk samples were taken from the second ears of *H. zea* infested corn ($F = 3.59$; df = 48; $P < 0.06$; $r = 0.26$) but was reduced when CryIA(b) protein concentration data from the Hawaii-grown plants was substituted in the analysis ($F = 2.34$; df = 11; $P < 0.16$; $r = 0.46$).

Discussion

Our data demonstrate that transgenic corn plants expressing CryIA(b) protein can significantly reduce *H. zea* larval feeding damage. Reduced *H. zea* feeding on transgenic corn could provide several important benefits to growers. Less damage to ears and kernels will result in increased grain yields and indirectly reduce the incidence of ear contamination from mycotoxin producing fungi. Within transgenic lines, both first and second ears have equivalent production of the CryIA(b) protein and should be equally protected from *H. zea* attack. Also, when transgenic corn is grown over large acreages, there is potential for area-wide suppression of *H. zea* populations with subsequently reduced damage to corn and other susceptible crops.

Field test results generally confirmed that corn lines with a higher concentration of CryIA(b) protein in silks were better protected from *H. zea* ear damage. However, the weak correlation between CryIA(b) protein levels and *H. zea* damage indicates that protein concentration alone may be inadequate for predicting expected *H. zea* protection. Avoidance, by *H. zea* larvae, of ear tissues with high concentrations of CryIA(b) protein could reduce the expected level of control. For example, several corn cultivars have high levels of the flavonol-C-glycoside "maysin" in silks which retard growth and prolong development of *H. zea* larvae (Wiseman et al. 1992). Corn high in maysin content may still be susceptible to *H. zea* larvae in the field because of other characters such as poor husk coverage (Wiseman and Widstrom 1992b). Similarly, neonate *H. zea* larvae, which are capable of considerable movement after egg hatch, might avoid feeding on the silks of transgenic corn in favor of kernels and other ear tissues, if these tissues have less CryIA(b) protein (see

Table 1. Corn earworm feeding damage to ears from 12 transgenic corn lines, isoline controls and CryIA(b) protein concentration in transgenic silks.

Mean cm ear penetration (SEM)		CryIA(b) protein (μg/g fresh weight)	
Transgenic*	Isoline control	Silk†	Silk‡
1.3 (0.4) a	4.9 (0.6)**	0.83	1.07 (4)
1.3 (0.4) a	6.4 (0.6)**	0.11	0.40 (1)
1.7 (0.5) a	7.0 (1.1)**	1.28	0.92 (4)
1.8 (0.4) a	4.8 (0.5)**	1.13	0.38 (2)
2.0 (0.5) a	5.8 (0.7)**	–	1.25 (1)
2.5 (0.3) ab	6.1 (0.5)**	0.07	0.32 (8)
2.8 (0.4) abc	6.0 (0.6)**	0.08	0.32 (5)
3.0 (0.4) abc	4.8 (0.8)	0.13	0.20 (6)
3.2 (0.5) bcd	5.5 (0.6)**	0.07	0.15 (8)
4.6 (0.6) bcd	5.2 (0.9)	0.09	0.13 (2)
4.9 (0.5) cd	5.2 (0.7)	0.13	0.00 (7)
5.3 (0.4) d	6.2 (0.6)	0.21	0.00 (1)

* Means within a column followed by the same letter are not significantly different ($P = 0.05$; HSD test [SAS Institute 1989]).

** Significantly different from transgenic line ($P < 0.05$; Student t-test).

† Material grown in Hawaii and sampled at the 2-4 day stage. Data represents one ELISA analysis of pooled sample material from 3 plants.

‡ Mean samples (2-4 day old) taken from secondary ears of plants infested with *H. zea* larvae at Jerseyville, IL. Number in parenthesis = sample size.

Gould and Anderson 1991, Gould et al. 1991). The relatively loose ear husks of the transgenic corn lines used for our field studies would not present a significant barrier to *H. zea* larvae moving from the outer silk to the inner ear.

Sublethal doses of CryIA(b) protein have an antibiosis-type effect on *H. zea* larvae resulting in prolonged development and severe stunting (S. R. S., unpublished data). Future studies should focus on the proportion of *H. zea* larvae completing development on transgenic corn and the reproductive capacity of surviving adults. These data will be valuable for predicting area-wide population suppression and in defining appropriate resistance management tactics. The effectiveness of sublethal concentrations of CryIA(b) protein for *H. zea* control should be enhanced in combination with other corn allelochemicals such as maysin. Optimal concentrations of a combination of chemical and protein growth inhibitors with distinct modes of action within the same corn line would be valuable in resistance management programs (Gould 1988).

Wiseman and Widstrom (1992a, b) reported on several lines of dent corn selected for resistance to *H. zea* larvae. The three most effective transgenic corn lines in the present study show *H. zea* resistance that is comparable, and probably superior, to the most resistant dent corn lines studied by these authors. Although, this level of protection would be a significant additional benefit for growers of transgenic corn, we believe that plant resistance to *H. zea* would be further enhanced by increasing CryIA(b) protein expression in silk and kernels, combining the "antibiosis" effect of the CryIA(b) protein with other resistance factors such as maysin, and increasing physical tolerance characters such as husk tightness.

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