Field Evaluation of Transgenic Tobacco Containing a *Bacillus thuringiensis* Insecticidal Protein Gene

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ABSTRACT Six transgenic tobacco lines expressing the CryIA(b) insecticidal protein from *Bacillus thuringiensis* var. *kurstaki* HD-1 were tested in 1990 to evaluate their efficacy under normal field conditions. Five of the six CryIA(b) lines tested kept laboratory-reared populations and natural populations of tobacco budworm, *Heliothis virescens* F., and tobacco hornworm, *Manduca sexta* L., below their respective economic threshold for the duration of the trial. Little or no economic damage from tobacco hornworm was sustained by any CryIA(b) line and only two lines sustained significant damage from tobacco budworm. Insect control with some CryIA(b) lines was equal to or better than control by standard Orthene and Dipel treatments. In addition, data obtained suggest there may be a positive interaction between CryIA(b) protein expression within a plant and parasitism of *Heliothis* spp.

KEY WORDS *Bacillus thuringiensis*, transgenic tobacco, field efficacy

ONE OF THE EARLIEST applications of genetic engineering to crop protection has been the production of insect-resistant plants. Several successful attempts at introducing insect resistance genes into crops have been reported by Barton et al. (1987), Fischhoff et al. (1987), Hilder et al. (1987), Vaeck et al. (1987), Delannay et al. (1989), Fuchs et al. (1990), and Perlak et al. (1990). In most of these reports, the investigators used one of the insecticidal crystal protein (ICP) genes derived from the soil bacterium *Bacillus thuringiensis*. *B. thuringiensis*, a gram-positive spore-forming bacterium, produces parasporal crystalline proteins that are toxic to a select spectrum of insects. The history, biology, and insecticidal properties of ICPs have been extensively reviewed elsewhere (Fast 1981, Aronson et al. 1986, Andrews et al. 1987, Hofte & Whitely 1989). The ICPs are characterized by their lack of toxicity to nontarget organisms, their high specific activity, and their efficacy. Microbial formulations of *B. thuringiensis* have been in existence for >30 yr and continue to dominate the biological control market (Wilcox et al. 1986, Rowe & Margaritis 1987).

Most reports on the efficacy of transgenic plants that express various ICPs pertain to data generated from laboratory and greenhouse experiments. Information regarding performance of such plants under field conditions is limited. Although a number of investigators have tested the performance of transgenic plants that express ICPs in the field, we are aware of only one published report of data (Delannay et al. 1989). In summer 1990, we conducted a comprehensive field experiment with transgenic tobacco plants containing a CryIA(b) insecticidal protein gene. The field test was designed to study the performance of different genetically characterized transformed lines, including a comparison of plants heterozygous and homozygous for the CryIA(b) gene. The performance of tobacco plants that express CryIA(b) was evaluated by comparison with wild-type plants untreated and treated with commercial insecticides. Transformed plants were also scored for resistance to laboratory versus natural populations of insects. The effect of abiotic stresses and insect parasitism was also examined. Levels of CryIA(b) insecticidal protein and mRNA were monitored weekly throughout the growing season. Here we present data on insect control and about protein expression as it relates to insecticidal activity.

Materials and Methods

Origin of Lines. Transgenic tobacco plants expressing the *Bacillus thuringiensis* CryIA(b) crystal protein gene from var. *kurstaki* HD-1 were generated by *Agrobacterium*-mediated leaf disk transformation (Horsch et al. 1987) of *Nicotiana tabacum* L. var. 'Havana 38'. The CryIA(b) gene was obtained from Martin Geiser (CIBA-GEIGY, Basel, Switzerland) (Geiser et al. 1986). Two truncated versions of the CryIA(b) gene were cloned into pCIB770, a binary Ti plasmid-derived plant transformation vector described by Rothstein et al. (1987), to create plasmids

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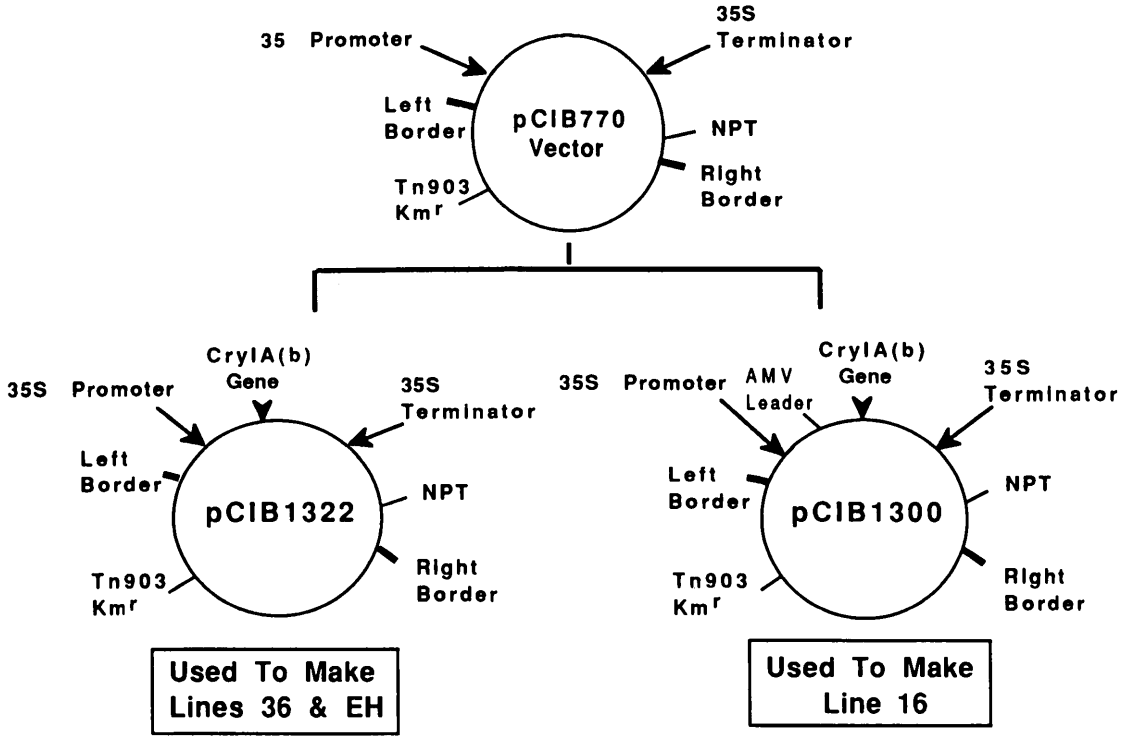

Fig. 1. *Bacillus thuringiensis* CryIA(b) gene constructs used to generate field test transgenic tobacco lines.

pCIB1300 and pCIB1322 (Fig. 1). Plasmid pCIB1322 contains 1,930 bp of the CryIA(b) gene coding for a 645 amino acid protein (~68,000 mol wt), with expression under control of the CaMV 35S promoter and 35S 3′ termination and polyadenylation sequences. Plasmid pCIB1300 is identical with the exception of a 40-bp untranslated leader sequence from the alfalfa mosaic virus (AMV) coat protein gene, which is inserted 5′ to the CryIA(b) AUG translational initiation codon. The AMV leader was added to determine whether it would enhance translation of the CryIA(b) mRNA (Jobling & Gehrke 1987).

Three tobacco parental lines (36, 16, and EH), derived from 'Havana 38' tobacco transformed with *Agrobacterium* strain A136, which contained plasmid pCIB1300 or pCIB1322, were selected for field evaluation after extensive laboratory and greenhouse testing confirmed their resistance to insects. Resistant plants were defined as those producing 90–100% mortality on first-instar tobacco hornworm, *Manduca sexta* L., and tobacco budworm, *Heliothis virescens* F., while sustaining minimal foliar damage. The transgenic tobacco lines 36 and EH were chosen from independent transformation events obtained using pCIB1322; line 16 was chosen from pCIB1300-derived transformants. Lines 36 and EH represent the highest and lowest CryIA(b) expressing lines, respectively, based on greenhouse bioassay and ELISA data (data not shown). Homozygous lines, designated 36S, 16S, and EHS, were generated by selfing the original transformed plants. Heterozygous lines (36H, 16H, and EHH) were generated by backcrossing the respective homozygous lines to 'Havana 38'. The six progeny lines were also characterized by Southern blot analysis and genetic segregation. All lines contained one copy of the CryIA(b) gene inserted at a single locus (data not shown). Polymerase chain reaction analysis indicated that all lines were positive for the CryIA(b) gene. Two control lines were included in the field test (i.e., the untransformed parent 'Havana 38' and pCIB10, a line transformed with the same plasmid as the CryIA(b) lines but lacking the CryIA(b) gene). These lines gave <5% insect mortality in greenhouse tests.

Plot Design. The field test was done on a private farm in Franklin County, NC. Plants were started in a greenhouse at the CIBA-GEIGY Agricultural Biotechnology Research Unit 6 wk before they were transplanted to the field in early May. All cultural practices, handled by a commercial grower, did not differ in any way from normal agronomic practices for commercial tobacco grown in North Carolina except for insecticide treatments.

The test consisted of 10 treatments: six CryIA(b) lines, one transformed-control line, one parental control line, one parental-control line treated with acephate (Orthene TIS), and one parental line treated with Dipel 2X (Orthene is a

registered trademark of Chevron Chemical; Dipel 2X is a registered trademark of Abbott Laboratories). Treatments were replicated 6 times and arranged in a randomized complete block design. Each replicated treatment or plot consisted of a single row (11.2 m long and 1.2 m apart) with 20 tobacco plants spaced 56 cm within the row. Each block was separated by a 4-m alleyway and the entire field was bordered by two rows of 'Havana 38'.

Because of the rapid growth and maturation of 'Havana 38' tobacco, the field test was divided into two simulated seasons. The time from transplant to 8 wk after transplant was designated season 1. Eight weeks after transplant, the plants were cut back to 3–4 leaf nodes and a single sucker was allowed to grow. The period of time from cutback to 7 wk after cutback was designated season 2.

CryIA(b) Protein Quantitation. All transgenic CryIA(b) lines were analyzed for expression of insecticidal protein. Polyclonal rabbit and goat antibodies purified by immunoaffinity and specific for the CryIA(b) protein were used to determine CryIA(b) protein levels in crude lysates of leaf samples by enzyme-linked immunosorbant assays (ELISA) (Clark et al. 1986). Leaf samples were taken from plants weekly. Sampling started 1 wk after transplantation and continued for 14 wk. Samples were obtained from three areas of the plant. Samples referred to as "top" were from 5.1–7.6 cm leaves in the bud region, "middle" samples were from leaves at nodes 5–11 aboveground, and "bottom" samples were from the first four nodes aboveground. Each week, three middle samples were taken from each of the six CryIA(b) lines and two control lines in four of the six replicate plots. In addition, one bottom and one top sample were taken from one plant in each of the four replicate plots. A total of 20 samples per line were tested by ELISA weekly.

Insect Infestation and Sampling (Season 1). To guarantee adequate insect pressure early in the season, the plants in the field were manually infested with laboratory-reared tobacco budworm, *Heliothis virescens* F., and tobacco hornworm, *Manduca sexta* L. Two weeks after they were transplanted to the field, the 10 interior plants of each plot were infested with neonate tobacco budworm. Five larvae were placed on each plant in the developing leaf–bud region. Two different blocks were infested per day for 3 d consecutively to ensure that adequate numbers of healthy larvae were available and that losses caused by weakened insects would be minimized. On each day of infestation, newly hatched larvae were obtained from Fred Gould (North Carolina State University). The first application of Orthene TIS and Dipel 2X was made 2 d after the last infestation. Orthene was applied at a rate of 1.1 kg (AI)/ha with a hand pump back-pack sprayer. Dipel 2X was applied as a 1% cornmeal bait. Approximately 1 g of the Dipel–cornmeal mixture was placed directly in the leaf bud by hand.

The plots were manually infested with neonate tobacco hornworm 4 wk after transplanting the tobacco. Five larvae were placed on the leaves in the middle of the plant to mimic a natural infestation. All blocks were infested the same day with larvae hatched from eggs obtained from Carolina Biological Supply Company (Burlington, NC). The second application of Orthene and Dipel was made 3 d after the infestation with tobacco hornworm.

Each week after infestation, all tobacco budworm and tobacco hornworm larvae were counted on the 10 interior plants of each plot. Larval size was characterized as small, medium, or large. Size estimates were more critical to evaluate the effects on tobacco hornworm because the economic threshold for this insect is five larvae measuring ≥2.54 cm (≥1 inch) (medium and large) per 50 plants (Southern 1986). Economic threshold for *Heliothis* spp. on tobacco is based on the number of infested plants, regardless of size of the larvae. The threshold used in this trial was five infested plants per 50 sampled (Southern 1986). Leaf damage was subjectively rated from 0 (no damage) to 3 (maximum damage: >60% of the leaves consumed by tobacco hornworm or plant topped by *Heliothis* spp.). A rating of 2 or 3 denoted economic damage. Data were analyzed by analysis of variance (ANOVA); and means were separated by least significant differences (LSD) (SAS Institute 1985).

Before the tobacco was cut back at the end of season 1, *Heliothis* spp. larvae were collected from each plot and maintained in the laboratory on artificial diet until adults emerged. The proportion of *H. virescens* and the corn earworm, *H. zea* (Boddie), was assessed to determine whether transgenic CryIA(b) plants would select for the less CryIA(b)-susceptible species *H. zea*. The *Heliothis* population collected was also examined for parasitism to determine if more insects were parasitized on transgenic CryIA(b) plants compared with control plants. Parasitism was scored by visual observation of adult parasite emergence.

Insect Infestation and Sampling (Season 2). No artificial insect infestation was needed for season 2. Three weeks after the plants were cut back, natural populations of tobacco hornworm and tobacco budworm moths began laying eggs throughout the field. Populations peaked 5 and 7 wk after cutback, respectively, for these species. Applications of Orthene and Dipel were made at 3 and 5 wk after cutback. Larval sampling and damage ratings were the same as described for season 1. As in season 1, *Heliothis* spp. larvae were collected at the end of season 2 from all plants within plots and brought back to the lab

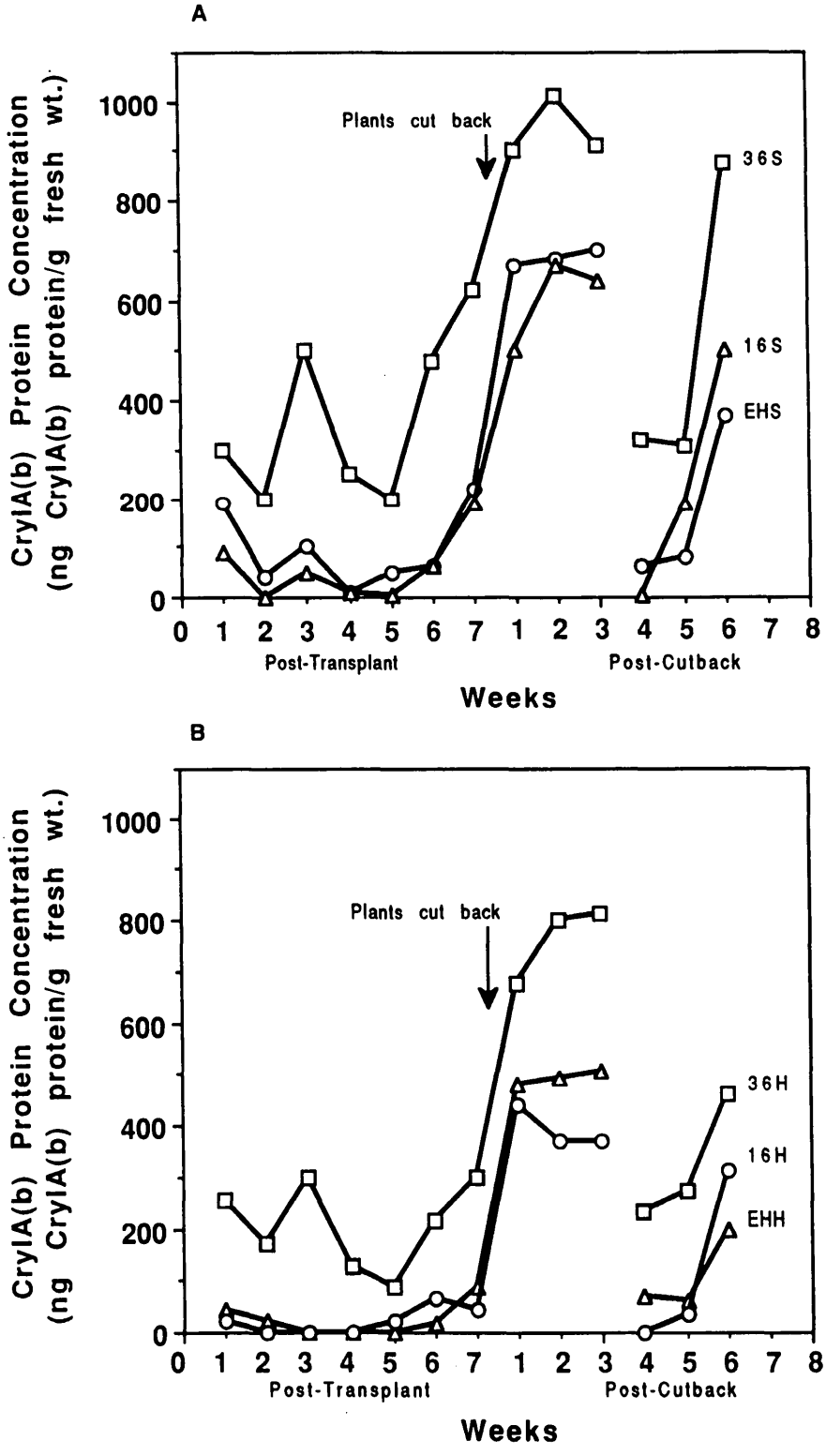

Fig. 2. Concentration of the CryIA(b) insecticidal protein in tobacco lines which are (A) homozygous and (B) heterozygous for the truncated CryIA(b) gene.

for rearing. Statistical significance was assessed by ANOVA and LSD test (SAS Institute 1985).

Results

CryIA(b) Protein Quantitation. CryIA(b) protein levels determined by ELISA in middle leaves of the three homozygous lines and the three heterozygous lines are shown in Fig. 2 A and B, respectively. ELISA values, typically measured in nanograms CryIA(b) per milligram soluble protein, were converted to nanograms CryIA(b) per gram leaf wet weight for graphical representation.

Lines 36 and EH are independent transformants derived from plasmid pCIB1322 and presumably differ only in genomic insertion site. Line 36S consistently expressed higher levels of CryIA(b) protein than did EHS during the 13 wk of seasons 1 and 2. Differences in CryIA(b) production in these two lines may indicate a genomic insertion site effect, sometimes referred to as position effect. Inclusion of the AMV 5′-untranslated leader in line 16 apparently did not increase CryIA(b) protein levels significantly. Because this was the only transformed line examined with the AMV leader, we could not differentiate between the effect of the leader and position effects on CryIA(b) production.

CryIA(b) protein levels increased in the developing leaves during the field test (Fig. 2). CryIA(b) protein levels in line 36S exceeded 1 μg/g fresh weight or 0.01% of the soluble protein in the middle leaves by 2 wk after cutback. Because plants were cut back 8 wk after transplantation, middle leaves sampled in weeks 1–3 after cutback were older leaves whereas leaves sampled in weeks 4–6 after cutback were from newer growth. The levels of CryIA(b) protein in middle leaves sampled during weeks 4–6 after cutback were equivalent to the levels in the middle leaves of younger plants at weeks 5–7 after transplant, which had comparable development.

The concentration of CryIA(b) protein in any plant at any given sampling was consistently higher in the homozygous lines than in the respective heterozygous lines (i.e., 36S versus 36H). In both homozygous and heterozygous lines, bottom leaves had higher CryIA(b) protein concentrations than middle or top leaves (data not shown). A 10-fold difference between bottom and top leaves was not uncommon and may have been the result of an accumulation of stable CryIA(b) protein in older tissue.

Plant Resistance: Season 1. The number of tobacco budworm-infested plants was significantly less in the 36S, 36H, and 16S plots than in control plots for the duration of season 1. Lines 16H, EHS, and EHH were significantly different than controls only at weeks 3 and 4 (Table 1). For lines EH and 16, in which expression of CryIA(b) protein was not as high as in line 36, efficacy between homozygous and heterozygous plants was different. Although these differences were not significant at some samplings, the trend was evident. There were no significant differences between the best three CryIA(b) lines (36S, 36H, and 16S) and Orthene in controlling tobacco budworm except at week 7. However, two applications of Orthene were required to achieve the same level of control provided by CryIA(b) protein expressed within the plant. Transgenic CryIA(b) lines were more effective than Dipel in controlling tobacco budworm for 2 of the 5 wk in season 1 (Table 1).

The number of tobacco hornworm larvae that attained a size of $\geq$2.54 cm per 50 plants is shown in Table 2. Five transgenic CryIA(b) lines were effective in controlling tobacco hornworm below the economic threshold. Line EHH did not control tobacco hornworm below the economic threshold and was not significantly different from control lines at week 7. No differences in the number of $\geq$2.54 cm tobacco hornworms were found on homozygous or heterozygous plants in lines 36 and 16. However, significantly fewer tobacco hornworm larvae were found on the homozygous versus heterozygous plants in the EH line with the lowest CryIA(b) expression.

During the course of season 1, all CryIA(b) lines except EHH provided greater tobacco hornworm control than Dipel 2X. This effect occurred because the placement of the Dipel–cornmeal bait was directed into the bud for tobacco budworm control and not broadcast over the entire plant. As was observed with tobacco budworm, Orthene treatments were as effective as transgenic CryIA(b) plants for control of tobacco hornworm.

Species composition of the *Heliothis* spp. population and comparison of parasitism on control versus CryIA(b) lines are summarized in Table 3. All *Heliothis* spp. collected at the end of season 1 were *H. virescens*, indicating little or no migration of *H. zea* into the field during this 7-wk period. *H. zea* typically migrates from maize and other crops into tobacco in late July–September. A higher proportion of *H. virescens* larvae were parasitized on the CryIA(b) lines compared with the control lines. Although not significant, this difference suggests a positive interaction between CryIA(b) expression in the plant and increased parasitism of *Heliothis* spp. Nonlethal concentrations of CryIA(b) retard growth and cause exposed insects to become sick and lethargic. In a field situation, this debilitating condition could allow parasites and predators to locate *Heliothis* larvae more effectively on CryIA(b) plants. An increase in parasitism could be expected if the presence of CryIA(b) in the transgenic plants caused a longer exposure of *Heliothis* spp. larvae to parasites by delaying the tendency to bore into leaf terminals, flower buds, or both. Most parasitized larvae collected were

Table 1. Efficacy of CryIA(b) tobacco in controlling first-season tobacco budworm

Line/treatment	Rate	No. budworm-infested plants/50 sampled after transplant, $\bar{x}$ ± SEM[a]			
		Week 4	Week 5	Week 6	Week 7
Dipel 2X	1 g/plant	15 ± 1a	5 ± 2c	2 ± 1bc	2 ± 1bc
'Havana 38'		14 ± 1a	15 ± 2a	5 ± 2ab	2 ± 1bc
pCIB10		14 ± 1a	8 ± 2bc	5 ± 2ab	5 ± 1b
EH H		6 ± 1b	13 ± 2a	7 ± 3a	2 ± 1bc
Orthene TIS	1 kg(AI)/ha	2 ± 1c	1 ± 1d	4 ± 1ab	5 ± 2b
16 S		2 ± 2c	1 ± 1d	0 ± 0c	1 ± 1c
16 H		1 ± 1c	7 ± 2bc	6 ± 2ab	2 ± 1c
EH S		1 ± 1c	7 ± 1bc	6 ± 2ab	1 ± 1c
36 H		0 ± 0c	0 ± 0d	0 ± 0c	0 ± 0c
36 S		0 ± 0c	0 ± 0d	0 ± 0c	1 ± 1c

First-season plants were manually infested with 5 neonate larvae/plant 2 wk after transplant.
Means within a column followed by the same letter are not significantly different (α = 0.05; LSD test [SAS Institute 1985]).
[a] Economic threshold is 5 budworm-infested plants per 50 sampled (Southern 1986).

on lines 16 and EH, which produce lower levels of CryIA(b) than line 36, particularly in the flower buds where the larvae were collected.

Plant Resistance: Season 2. Results in season 2 were very similar to those obtained in season 1. The 36S and 36H lines maintained complete tobacco budworm control for the duration of the trial (Table 4). Significantly fewer plants in lines 16S, 16H, EHS, and EHH were infested with tobacco budworm than in the controls at weeks 3 and 4 of season 2. At weeks 5 and 6 of season 2, when most of the tobacco budworms were feeding on flower buds and petals, the number of infested plants in these lines was greater than the economic threshold and in some cases was equal to the controls. This increase in the larval numbers suggests that CryIA(b) protein concentration may not have been high enough in buds and petals of lines 16 and EH to kill tobacco budworms. Concentrations of CryIA(b) protein in the flower parts of line 36 were sufficient to kill all tobacco budworms. ELISA data suggest that CryIA(b) protein concentration is higher in older leaf tissue than in new leaf tissue and can be up to 10 times higher in older leaf tissue than in flower buds (data not shown). This difference was not evident in the insecticidal activity of the 36S and 36H lines because the CryIA(b) protein concentration was high enough in all infested plant tissue to kill tobacco budworm larvae.

Although the plants were exposed to very high tobacco hornworm pressure, as indicated by the number of insects on the control lines, all CryIA(b) lines could maintain this pressure considerably below the economic threshold (Table 5). In most weeks, not a single larva of ≥2.54 cm was found on any CryIA(b) expressing plant.

At certain weeks during season 2, CryIA(b) lines appeared to be more effective than Orthene for control of tobacco budworm and tobacco hornworm (Table 4 and 5). Higher efficacy of the CryIA(b) plants most likely reflects poor timing of Orthene applications rather than a lack of efficacy of Orthene. The earlier Orthene is applied after an infestation, the more effective it will be. Constitutive production of CryIA(b) protein within the plant obviates the need for precise timing of chemical applications.

Of the *Heliothis* species collected at the end of season 2, 75 and 68% were *H. virescens*, and 25

Table 2. Efficacy of CryIA(b) tobacco in controlling first-season tobacco hornworm

Line/treatment	Rate	No. hornworms (≥2.54 cm)/50 plants after transplant, $\bar{x}$ ± SEM[a]		
		Week 5	Week 6	Week 7
pCIB10		95 ± 5a	137 ± 13a	17 ± 2a
'Havana 38'		91 ± 7a	89 ± 4b	5 ± 2b
Dipel 2X	1 g/plant	36 ± 4b	17 ± 5c	17 ± 4a
EH H		4 ± 1c	10 ± 2c	12 ± 4a
16 H		1 ± 1c	0 ± 0d	0 ± 0b
EH S		0 ± 0c	1 ± 1d	0 ± 0b
Orthene TIS	1 kg(AI)/ha	0 ± 0c	0 ± 0d	0 ± 0b
16 S		0 ± 0c	0 ± 0d	0 ± 0b
36 H		0 ± 0c	0 ± 0d	0 ± 0b
36 S		0 ± 0c	0 ± 0d	0 ± 0b

Means within a column followed by the same letter are not significantly different (α = 0.05; LSD test [SAS Institute 1985]).
[a] Economic threshold is 5 hornworms (≥2.54 cm) per 50 plants (Southern 1986).

Table 3. Effect of transgenic CryIA(b)-tobacco on *Heliothis* parasitism and species composition

Line	% Parasitism (*n*)	% Species composition	
		H. virescens	*H. zea*
	Season 1		
CryIA(b)[a]	54.7 (106)	100	0
Control[b]	41.7 (60)	100	0
	Season 2		
CryIA(b)	56.8 (103)	68	32
Control	44.4 (54)	75	25

[a] Larval collections from all 6 CryIA(b) lines combined.
[b] Larval collections from 'Havana 38' and pCIB10 lines combined.

Table 4. Efficacy of CryIA(b) tobacco in controlling second-season *Heliothis* spp.

Line/treatment	Rate	No. budworm-infested plants/50 sampled after cutting back, $\bar{x} \pm$ SEM[a]			
		Week 3	Week 4	Week 5	Week 6
pCIB10		14 ± 2a	6 ± 2a	12 ± 2ab	12 ± 3b
'Havana 38'		13 ± 1a	6 ± 2a	10 ± 2b	18 ± 3ab
Dipel 2X		9 ± 1ab	6 ± 3a	15 ± 4a	22 ± 4a
Orthene TIS		7 ± 1b	8 ± 2a	12 ± 2ab	9 ± 2bc
EH H		3 ± 1c	5 ± 2ab	10 ± 2b	18 ± 3ab
EH S		1 ± 1c	2 ± 1bc	6 ± 2c	17 ± 2ab
16 S		1 ± 1c	1 ± 1c	8 ± 3c	6 ± 2c
16 H		0 ± 0c	1 ± 1c	9 ± 1bc	8 ± 3bc
36 H		0 ± 0c	0 ± 0c	1 ± 1d	0 ± 0d
36 S		0 ± 0c	0 ± 0c	0 ± 0d	0 ± 0d

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; LSD test [SAS Institute 1985]).
[a] Economic threshold is 5 budworm infested plants per 50 sampled (Southern 1986).

and 32% were *H. zea* from control and CryIA(b) lines, respectively (Table 3). These data did not indicate that the CryIA(b) plants were selecting for one species over another. If the predominant *Heliothis* species found on CryIA(b) plants was *H. zea*, CryIA(b) protein levels were probably high enough to control the more CryIA(b)-sensitive species, *H. virescens*, but not high enough to control *H. zea*. This phenomenon might lead to an artificial selection pressure against *H. virescens* in favor of *H. zea*. However, levels of CryIA(b) protein were high enough in the CryIA(b) lines to control both species.

The level of parasitism appeared to follow the same pattern observed in season 1. Although a higher proportion of *Heliothis* larvae were parasitized on CryIA(b) lines compared with control lines (Table 3), the difference was not significant.

CryIA(b) Protein Concentration–Economic Damage Comparison. At any sampling, CryIA(b) lines that had few or no insects feeding on them were also the lines that produced higher levels of CryIA(b) protein in the leaves. Summary data of this relationship for tobacco hornworm and tobacco budworm in season 1 and season 2 are shown in Tables 6 and 7, respectively. Mean CryIA(b) protein concentration (Table 6) was calculated by averaging all weekly ELISA values for the middle leaves, the area where tobacco hornworm typically feeds. Lines with higher levels of CryIA(b) protein produced in the middle leaves sustained less economic damage than either lines with lower levels of CryIA(b) or the control lines, pCIB10 and 'Havana 38' (Table 6). Data for tobacco budworm were similar (Table 7). The mean CryIA(b) protein concentration in this table was calculated by averaging the ELISA values from top leaf samples, the area where tobacco budworm typically feeds. Higher levels of CryIA(b) confer increasing levels of protection to tobacco budworm feeding damage. Both sets of data demonstrate that <50 ng of CryIA(b) protein/g fresh weight in top or middle leaves are required to control tobacco budworm and tobacco hornworm larvae below the economic threshold.

Discussion

The results of this field trial demonstrate the effectiveness of transgenic tobacco plants that express the CryIA(b) protein in controlling agronomically important pests. A single copy of the CryIA(b) gene provided sufficient levels of CryIA(b) insecticidal protein to protect plants at all stages of development. Expression levels of

Table 5. Efficacy of CryIA(b) tobacco in controlling second-season tobacco hornworm

Line/treatment	Rate	No. hornworms (≥2.54 cm)/50 plants after cutting back, $\bar{x} \pm$ SEM[a]			
		Week 3	Week 4	Week 5	Week 6
pCIB10		15 ± 4a	30 ± 6a	143 ± 20a	97 ± 16a
'Havana 38'		4 ± 2b	7 ± 3b	45 ± 9bc	58 ± 6b
Dipel 2X	1 g/plant	2 ± 1b	5 ± 2bc	37 ± 8b	49 ± 8b
Orthene TIS	1 kg (AI)/ha	1 ± 1b	3 ± 1bc	14 ± 1c	2 ± 1c
16 H		0 ± 0b	0 ± 0c	1 ± 1d	1 ± 1c
EH H		0 ± 0b	0 ± 0c	0 ± 0d	0 ± 0c
EH S		0 ± 0b	0 ± 0c	0 ± 0d	0 ± 0c
16 S		0 ± 0b	0 ± 0c	0 ± 0d	0 ± 0c
36 H		0 ± 0b	0 ± 0c	0 ± 0d	0 ± 0c
36 S		0 ± 0b	0 ± 0c	0 ± 0d	0 ± 0c

Means within a column followed by the same letter are not significantly different ($\alpha = 0.05$; LSD test [SAS Institute 1985]).
[a] Economic threshold is 5 hornworms (≥2.54 cm per 50 plants (Southern 1986)).

Table 6. Relationship between CryIA(b) protein concentration and economic damage caused by tobacco hornworm

Line	CryIA(b) protein concn. (ng/g), $\bar{x}$ ± SEM[a]	% plants with economic damage
	Season 1	
'Havana 38'	0 ± 0	87
pCIB10	0 ± 0	98
EH H	21 ± 8	8
16 H	27 ± 10	0
EH S	58 ± 20	0
16 S	95 ± 28	0
36 H	208 ± 29	0
36 S	354 ± 58	0
	Season 2	
'Havana 38'	0 ± 0	95
pCIB10	0 ± 0	100
16 H	103 ± 27	5
EH H	110 ± 58	5
16 S	154 ± 68	0
EH S	199 ± 96	0
36 H	274 ± 45	0
36 S	458 ± 96	0

[a] CryIA(b) protein concentrations are season averages of middle-leaf samples.

Table 7. Relationship between CryIA(b) protein concentration and economic damage caused by *Heliothis* spp.

Line	CryIA(b) protein concn. (ng/g), $\bar{x}$ ± SEM[a]	% plants with economic damage
	Season 1	
'Havana 38'	0 ± 0	24
pCIB10	0 ± 0	23
EH H	9 ± 4	25
16 S	9 ± 7	0
16 H	14 ± 7	8
EH S	28 ± 4	13
36 H	80 ± 36	0
36 S	152 ± 67	0
	Season 2	
'Havana 38'	0 ± 0	60
pCIB10	0 ± 0	52
EH H	4 ± 1	33
16 S	12 ± 10	2
16 H	16 ± 8	8
EH S	32 ± 20	0
36 H	112 ± 54	0
36 S	159 ± 39	0

[a] CryIA(b) protein concentrations are season averages of upper-leaf samples.

CryIA(b) protein varied in the different transformed lines and led to a wide range of CryIA(b) protein levels. The production of up to 1 μg CryIA(b) protein/g leaf had no apparent deleterious effect on the plants as determined by visual inspections of plant phenotypes.

To our knowledge, this is the first field trial to test homozygous and heterozygous CryIA(b) plants from the same transformation event for differences in CryIA(b) expression and insect resistance. Our data demonstrate that heterozygous plants can be generated that express sufficient levels of CryIA(b) protein to protect the plant against economic damage. This result has particularly important implications for engineering hybrid crops.

Pressure from *Heliothis* spp. and *Manduca* spp. was extremely high, particularly from the natural infestation during the second season. This pressure is evident in the amount of economic damage sustained by the control lines. With no insect control from Orthene, Dipel, or CryIA(b) protein, the pCIB10 control line sustained 23 and 98% economic damage from tobacco budworm and tobacco hornworm, respectively in season 1, and 52 and 100% economic damage in Season 2 (Tables 6 and 7). Most of the late-season pressure came from tobacco hornworm. If left unchecked, this insect can cause considerable damage when it reaches the fourth and fifth instars. Every instar of the tobacco hornworm was found in the control plots, and the majority were L4 and L5. Because larvae did not attain this size in any of the transgenic CryIA(b) plots, damage was considerably less than in the control plots. In the transgenic CryIA(b) plots, particularly 36S and 36H, damage mirrored that observed in greenhouse tests, in which first instars died after making very small holes in the leaves.

In general, the relationship between the amount of CryIA(b) protein expressed in the plant and the level of protection from insect damage was positive. This association was difficult to quantify because all lines in this trial were very efficacious. For data to show significant differences, lines that express very low levels of CryIA(b) would have to be compared with the high-expression lines. No lines that produced low levels of CryIA(b) protein were chosen for these tests, making direct comparisons in a field situation difficult.

As more transgenic plants are planted in the field, a much-needed data base will be generated on how well laboratory and greenhouse performance predict field performance and how these plants can fit into an insect control scheme. The performance of the transgenic tobacco lines tested in this trial demonstrated that greenhouse performance was an accurate predictor of field efficacy. The fact that only two living *Heliothis* spp. larvae were found on 36S and 36H for the entire 13 wk of the trial is a clear indication of the commercial potential of genetically engineered plants that express the CryIA(b) protein.

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