Laboratory and Field Evaluations of Transgenic Soybean Exhibiting High-Dose Expression of a Synthetic *Bacillus thuringiensis cry1A* Gene for Control of Lepidoptera

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ABSTRACT Transgenic lines of soybean, *Glycine max* (L.) Merrill, expressing a synthetic *cry1A* gene (*tic107*) from *Bacillus thuringiensis* (Bt), were evaluated in screenhouse and conventional field trials for efficacy against lepidopteran pests. In screenhouse trials, Bt soybean and negative checks (isogenic segregants and parental lines) were evaluated against *Anticarsia gemmatalis* Hübner and *Pseudoplusia includens* (Walker) in the United States and against *A. gemmatalis*, *Epinotia aporema* (Walsingham), *Rachiplusia nu* (Guenée), and *Spilosoma virginica* (F.) in Argentina. Bt soybean exhibited virtually complete efficacy against each of these pests, whereas negative checks suffered significant damage. Bt soybean and negative checks also were evaluated in conventional trials against native populations of *A. gemmatalis* and *P. includens* in the southeastern United States. Each of these insects caused significant damage to negative checks in one or more locations, whereas Bt soybean exhibited virtually complete efficacy against these pests. In the laboratory, lyophilized leaf tissues from Bt soybean incorporated in artificial diet at a concentration representing a 25-fold dilution of fresh tissue caused complete mortality of *A. gemmatalis* and near complete mortality of *P. includens* neonates after 11 d, whereas mortality on negative checks did not exceed 10% for either insect. Average TIC107 expression approached or exceeded 50 μg/g fresh weight at V3 stage of growth and 200 μg/g by R6 stage of growth. These results demonstrate that expression of TIC107 in soybean can not only achieve highly efficacious control of several lepidopterans under field conditions but also provide a high dose for effective insect resistance management.

RESUMEN Se evaluó resistencia a plagas lepidópteras de lineas de soja transgénicas, *Glycine max* (L.) Merrill, que expresan un gen *cry1A* sintético (*tic107*) de *Bacillus thuringiensis* (Bt), en ensayos a campo, con y sin jaulas. En ensayos con jaulas, las sojas Bt y los testigos negativos (segregantes isogénicos y líneas parentales) fueron evaluadas frente a *Anticarsia gemmatalis* Hübner y *Pseudoplusia includens* (Walker) en los EE.UU. de A. y frente a *A. gemmatalis*, *Epinotia aporema* (Walsingham), *Rachiplusia nu* (Guenée), y *Spilosoma virginica* (F.) en Argentina. La soja Bt exhibió una eficacia virtualmente total frente a cada una de las plagas, mientras que los testigos negativos sufrieron un significativo daño. La soja Bt y los testigos negativos también fueron evaluados en ensayos convencionales frente las poblaciones naturales de *A. gemmatalis* y *P. includens* en el sudeste de los EE.UU. de A. Cada uno de estos insectos causó daño significativo a los testigos negativos en una o más localidades, mientras que la soja Bt mostró una eficacia virtualmente total a dichas plagas. En el laboratorio, tejidos foliares liofilizados provenientes de soja Bt incorporados en dieta artificial a una concentración que representa una dilución de 25 veces la del tejido fresco, causó total mortalidad de neonatas de *A. gemmatalis* y casi completa mortalidad de neonatas de *P. includens*, luego de 11 días, mientras que la mortalidad en los testigos negativos no excedió el 10% en ambos insectos. La expresión de TIC107 promedió se aproximó o excedió los 50 μg/g de peso fresco en estado V3 de crecimiento

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y los 200 μg/g en estado R6. Estos resultados demuestran que la expresión de TIC107 en soja no sólo puede lograr un control altamente eficaz de varios lepidópteros en condiciones de campo, sino también proveer una alta dosis para un efectivo manejo de resistencia de insectos.

KEY WORDS *Bacillus thuringiensis*, high dose, Lepidoptera, resistance management, transgenic soybean

SOYBEAN, *Glycine max* (L.) Merrill (Fabaceae: Phaseoleae), is a major agricultural crop in the Western Hemisphere, with >80% of global production occurring in the United States, Brazil, and Argentina (United Soybean Board 2000). Soybean crops in more southern latitudes of this region (southeastern United States to Argentina) are subject to pressure by several lepidopteran insect pests (Turnipseed and Kogan 1976, Higley and Boethel 1994, Aragón et al. 1997). *Anticarsia gemmatalis* Hübner, velvetbean caterpillar, is the most important of these, occurring throughout much of this region, whereas *Pseudoplusia includens* (Walker), soybean looper; *Epinotia aporema* (Walsingham), "barrenador del brote"; and *Rachiplusia nu* (Guenée), "oruga medidora del girasol", are important in more restricted areas. Other lepidopterans of sporadic importance include *Helicoverpa zea* (Boddie), bollworm; *Spodoptera frugiperda* (J.E. Smith), fall armyworm; *Elasmopalpus lignosellus* (Zeller), lesser cornstalk borer; and *Spilosoma virginica* (F.) "gata peluda norteamericana".

Chemical insecticides are commonly used for controlling lepidopteran infestations in soybean but are not always effective. The cryptic habits of *E. aporema* larvae protect them from insecticidal sprays, making high rates and careful timing of systemic insecticide applications necessary for effective control (Aragón et al. 1997). *Pseudoplusia includens* has developed resistance to every synthetic class of insecticide used against it (Thomas and Boethel 1994), and resistance to pyrethroids is widespread across the southern United States (Felland et al. 1990, Leonard et al. 1990). Insecticides remain effective against *A. gemmatalis;* however, infestations can quickly reach damaging levels and cause economic loss if insecticides are not applied promptly. Alternative control strategies such as biological insecticides and natural enemies are available but not widely used (Luttrell et al. 1998, Moscardi 1999), and efforts to develop elite insect-resistant soybean lines with high yield and desirable agronomic characteristics through conventional breeding of germplasms exhibiting endogenous insect resistance have not been successful (Boethel 1999).

Advances in crop biotechnology offer a promising alternative to chemical insecticides for control of lepidopteran pests in soybean. Since their introduction in 1996, transgenic crop varieties expressing δ-endotoxins (Cry proteins) from *Bacillus thuringiensis* (Bt) have become important tools for effective pest management. The adoption of Bt cotton, *Gossypium* spp., has not only enabled more effective management of lepidopteran pests but also significantly reduced chemical insecticide use (Perlak et al. 2001, Carrière et al. 2003). Likewise, the adoption of Bt corn, *Zea mays* L. (Armstrong et al. 1995), has reduced the impact of lepidopteran stalk borers (Pilcher and Rice 2003). Bt soybean varieties have yet to be commercialized, although experimental lines have been developed. The first report of such, expressing a native *cry1Ab* gene, deterred *A. gemmatalis* larval feeding and growth (Parrott et al. 1994). Another (Jack-Bt), expressing higher levels of a synthetic *cry1Ac* gene, caused complete *A. gemmatalis* larval mortality and significantly reduced *P. includens* and *H. zea* larval survival and feeding in laboratory bioassays (Stewart et al. 1996). This line also exhibited efficacy against these pests in artificially infested field cages (Walker et al. 2000), although damage did exceed economic injury thresholds (Sullivan and Boethel 1994) in a few trials. This was attributed to migration of older, presumably more tolerant, larvae from adjacent non-Bt plots. Nevertheless, it raises questions about the ability of Jack-Bt soybean to provide consistent control across multiple geographies and its suitability in a "high dose/refuge" insect resistance management strategy (Gould 1994).

We report transgenic Bt soybean lines expressing a synthetic *cry1A* gene that effectively control a variety of lepidopteran pests across multiple geographies. In addition, we provide direct evidence that they express the Cry protein at levels constituting high dose against *A. gemmatalis* and *P. includens* and indirect evidence of high dose expression against other lepidopterans.

Materials and Methods

Plant Genotypes. The transgenic soybean lines used in these studies were created using *Agrobacterium*-mediated DNA transfer (Hinchee et al. 1988) into soybean ('A5547'). Among the resultant R_0 plants, three lines (19459-55, 19478-8, and 19487-35) were determined to contain one intact copy of the Bt gene *tic107*, a synthetic *cry1A* construct similar to Cry1Ac (Fischhoff and Perlak 1995), as confirmed by Southern blot analyses (data not shown). The 3.5-kb *tic107* coding sequence is controlled by the ribulose-1,5-bisphosphate carboxylase (Rubisco) small subunit promoter (ArabSSU1A) from *Arabadopsis thaliana* (L.) Heynh., with translocation of the precursor protein to the chloroplast under the direction of the N-terminal stromal-import sequence also from ArabSSU1A (Almeida et al. 1989, Wong et al. 1992). All three lines lack marker genes or other plasmid DNA sequences outside of the transfer DNA (T-DNA) region, again as confirmed by Southern blot analyses

(data not shown). Isogenic Bt-negative lines were derived for each line through Mendelian segregation.

Protein Expression. TIC107 expression was evaluated in field-grown, homozygous R_4 plants of two Bt lines (19459-55 and 19487-35) and their negative isolines planted at three locations (Galena, MD; Leland, MS; and Loxley, AL) during the 2002 growing season. Leaf tissues were collected at V3, R1 (R4 at Galena), and R6 stages of plant growth (Fehr et al. 1971) by picking the center leaflet of the youngest fully expanded trifoliate from 10 randomly selected plants within three plots of each Bt line and one plot of each negative isoline. For protein expression analysis, 100 mg of crushed, frozen leaf tissue was combined with 10 ml of extraction buffer (50 mM sodium carbonate/bicarbonate + 2 mM dithiothreitol, 0.07% Tween 20) in a 15-ml polyethylene tube. Eight 6.35-mm chrome-steel beads (BioSpec Products Inc., Bartlesville, OK) were added to the tubes and vigorously shaken in a Harbil 5G HD case mixer (Fluid Management, Wheeling, IL) for 7 min. The extract was separated from the solid phase using a serum filter (Fisher, Pittsburgh, PA), and 100 μl of extract was treated with trypsin (Calbiochem, La Jolla, CA) equivalent to 540 USP units/ml and incubated at 37°C for 30 min. The protease activity was stopped with 0.125 μM phenylmethylsulfonyl fluoride (PMSF) from a stock solution of 50 mM PMSF in 2-propanol. Samples were diluted 1:30 and 1:90 with a Tris-borate buffer (TBA) containing 100 mM Tris base, 100 mM $Na_2B_4O_7$ * $10H_2O$, 5 mM $MgCl_2$ * $6H_2O$, 0.05% Tween 20, HCl to pH 7.8, and, just before use, 0.2% L-ascorbic acid dissolved into solution, before loading on assay plates.

Protein expression was determined by indirect enzyme-linked immunosorbent assay (ELISA) (Crowther 1995). A Cry protein standard, comparable with the Cry1Ac encoded by Bt subsp. *kurstaki* strain HD-73 expressed and purified from *Escherichia coli* fermentation, was lyophilized, dissolved in 50 mM sodium bicarbonate (pH 9.6), subjected to trypsinization as described for sample treatment, and serially diluted in TBA to eight concentrations ranging from 32 to 0.25 ng/ml to generate a standard curve for quantitation of the expressed TIC107. For ELISA, 96-well MaxiSorp Nuncimmuno plates (NUNC A/S, Roskilde, Denmark) were coated with 200 μl/well monoclonal IgG Ab1 (M19N4A6, from mouse) diluted 1:4,800 in coating buffer (50 mM carbonate/bicarbonate and 150 mM NaCl), incubated overnight at 4°C, washed three times with PBST (0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, 0.0027 M KCl, 0.07% Tween 20, pH 7.4), blocked with 200 μl/well PBSTO (1× PBST, 0.5% oval albumin) at room temperature (RT) for 2 h, and washed one time with PBST. Plates were loaded with 100 μl/well of sample (three replicates per plate for standard, test samples, and positive controls; eight per plate for negative controls [A5547 leaf extracts] and TBA buffer blanks), incubated at RT for 2 h and washed three times with PBST. Plates were loaded with 200 μl/well of HD-73 rabbit polyclonal antibody (SDI, Newark, DE), diluted 1:1,000 with PBSTO, incubated at RT for 1.5 h, and washed three times with PBST. Bound polyclonal IgG was detected with 200 μl/well of donkey anti-rabbit IgG-horseradish peroxidase conjugate (Amersham Biosciences Inc., Piscataway, NJ) diluted 1:3,000 with PBSTO. Plates were washed three times with PBST, and then 100 μl/well TMB peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, MD) was added and incubated at RT in the dark for 4 min. Reactions were stopped with phosphoric acid, and absorbance was read at 450 nm in a Spectra Max plate reader by using SOFTmax PRO version 3.0 software (Molecular Devices, Sunnyvale, CA). Expression data were subjected to analysis of variance (ANOVA) by using JMP software version 5.1.1 (SAS Institute 2004) to determine significant sources of variability among location, line, and replicate at the 0.05 probability level (P). Significant differences among means were determined using the Tukey–Kramer test (Kramer 1956) at $P = 0.05$.

2002 Argentina Screenhouse Trials. Pooled progeny from homozygous and hemizygous R_1 (first generation after transformation) plants of Bt lines 19478-8 and 19487-35 were evaluated in screenhouse trials at the Monsanto Agronomy Center, Fontezuela, Buenos Aires Province, and the Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria (INTA-EEA), Oliveros, Santa Fe Province. Negative-expressing R_2 plants were culled from Bt-positive plots, leaving only hemizygous and homozygous Bt-positive plants in those plots, and the parental 'A5547' served as a negative check. Two identical randomized complete blocks (RCB) with four replicates per entry (soybean line) were established at each location under 16 by 18-mesh nylon insect screen covers supported by iron tubing frames measuring 11.0 m in length by 7.5 m in width by 2.0 m in height. Seed was planted 8–22 January 2002 by hand at a rate of ≈25 seeds per meter row in plots measuring one row by 2.0 m with 1.0-m spacing, 80-cm alleys, and no borders/buffers. Plots were weeded by hand with a hoe as necessary during the trial period. At 37–64 d after planting (dap), plants in the Bt-positive plots were subjected to expression analysis by using a Monsanto GeneCheck Lab test kit and culled if a negative result was obtained, leaving only homozygous and hemizygous Bt-positive plant genotypes within those plots. One screenhouse at each location was infested with *A. gemmatalis* (40–800 adults and 20–180 second to third instars per row) two to three times (49–62 dap) or *E. aporema* (200–1,000 adults) two to four times (41–84 dap). Insects were obtained from laboratory colonies at INTA-EEA in Oliveros. Adult *A. gemmatalis* also were obtained in Oliveros from light traps in soybean fields. Adult food was provided by absorbent cotton saturated with a sucrose/honey solution (Nipagin added as a mold inhibitor) in plastic boxes throughout the house. Larval incidence of *A. gemmatalis* was evaluated at R4 stage of plant growth (62–65 dap) by beating the center 60 cm of each row over a vertical beat sheet and counting the number of larvae. Damage was assessed at R5 stage of growth (67–76 dap) by estimating percentage of defoliation

on 40 randomly selected leaves from both the upper and the lower portions of the canopy in each plot. Larval incidence of *E. aporema* was evaluated at R5 stage of growth (64–84 dap) by counting the number of live larvae on 10 randomly selected plants in each plot. Damage was assessed concurrently on the same plants by counting the number exhibiting damage. In all trials, pod production was evaluated at maturity (112–133 dap) by counting the number of pods on 10 random plants in each plot, and yield was measured in the Oliveros trials by harvesting the entire row in each treatment and weighing the seed. Data on larval incidence, damage, pod production, and yield were subjected to ANOVA to determine significant sources of variability among location, line, and replicate at the 0.05 probability level. Significant differences among means were determined using the Tukey–Kramer test at $P = 0.05$.

2002 U.S. Screenhouse Trials. Homozygous R_4 plants of Bt lines 19459-55 and 19487-35 were evaluated in screenhouse trials at the Monsanto Agronomy Centers in Jerseyville, IL, and Monmouth, IL. Negative isolines and/or the parental 'A5547' served as negative checks. Two identical randomized complete blocks (RCB) with four replicates per entry (soybean line) were established at each location under 16 by 18-mesh nylon insect screen covers supported by aluminum-tubing frames measuring 15.5 m in length by 9.1 m in width by 2.9 m in height at the center. Seed was planted 1–4 June 2002 with a cone planter at a rate of 16.4–27.3 seeds per meter row in plots measuring one row by 2.0 m with 76.2-cm spacing, 50-cm alleys, and no borders/buffers. Weeds were controlled with preplant herbicides labeled for soybean use or hand hoeing as necessary during the trial period. At each location, screenhouses were infested by placing 2,000 *A. gemmatalis* pupae (obtained from a laboratory colony at the Monsanto Technology Center, Union City, TN) in the house at V3 (27–32 dap) and R3 (79–83 dap) stages of growth or 1,000 *P. includens* pupae (reared from eggs obtained from a laboratory colony at the University of Georgia Coastal Plain Experiment Station, Tifton, GA) at V4 (36–40 dap) and R3 (72–76 dap) stages of growth. Adult food was provided by Styrofoam cups containing absorbent cotton saturated with 10% sucrose solution. Damage was assessed at 7-d intervals beginning with the first infestation and ending with the cessation of larval activity or onset of plant senescence by estimating percentage of defoliation in each plot. Damage data were analyzed as described previously.

2002 U.S. Field Trials. Homozygous R_4 plants of Bt lines 19459-55 and 19487-35 were evaluated in open field trials at the Monsanto Agronomy Centers in Leland, MS, and Loxley, AL; the University of Georgia Coastal Plain Experiment Station, Tifton; Louisiana State University (LSU) Dean Lee Research Station, Alexandria; and LSU St. Gabriel Research Station, St. Gabriel. Negative isolines and/or the parental 'A5547' served as negative checks. A split-plot RCB design was used, with main plot treatments consisting of soybean entry (three replicates each), and subplot treatments consisting of prophylactic insecticidal sprays versus no sprays. This allowed for comparison of larval incidence, damage, and yield in soybean lines when exposed to endemic lepidopteran pest populations versus when protected from such. Four-row buffers (parental 'A5547') separated the four-row subplots to prevent spray drift, and borders ('A5547') consisted of four or more rows on each side and typical plot lengths on the front and back of the test block. Seed was planted 4–11 June 2002 with a cone planter in 9.14–10.0-m rows spaced 91.4–96.5 cm apart at a rate of 23–25 seeds per m row. Weeds were controlled with preemergence and postemergence herbicides labeled for use on soybean. Insecticides were applied to "sprayed" subplots to prevent damage by lepidopteran pests, whereas "unsprayed" subplots received no such treatments. In some cases, insecticide treatment of the entire test block (all plots, buffers, and borders) was required to prevent significant damage by nonlepidopteran pests (e.g., stink bugs, alfalfa hopper, and bean leaf beetle). Herbicides and insecticides used varied across locations but were limited to commercial products labeled for their intended use. Larval incidence of lepidopteran pests, damage, and plant growth stage were recorded periodically (generally every 7–10 d) from R1 to R2 stage of growth until late R6 to R7 stage of growth. Larval incidence was determined by beating plants from the two middle rows in each subplot over a 0.91-m ground cloth. Two samples were taken from each plot during each observation, with larval counts for each species converted to larvae per meter of row. At maturity, the two middle rows of each subplot were harvested with an experimental plot harvester. Yields were calculated after correcting seed weight to 13% moisture. Data on larval incidence, damage, and yield were analyzed as described previously.

2003 Argentina Screenhouse Trials. Homozygous R_5 plants of all three Bt lines were evaluated in screenhouse trials at Fontezuela and Oliveros. Negative isolines and the parental 'A5547' were included in each trial as negative checks. Essentially, the same methods and experimental design were used as in the 2002 Argentina trials except as noted here. Trials were planted 10–21 January 2003 at a rate of 30 seeds per meter row, with no culling of negative plants required. Screenhouses were infested with *R. nu* at both locations (50–60 first to second instars per row) at V5 to V6 stage of growth (25–41 dap) and at Oliveros (350 first to second instars per row) at R5 stage of growth (70 dap). Larvae were reared from eggs obtained from adults collected at light traps at Oliveros and maintained in oviposition cages in the laboratory. An additional screenhouse at Oliveros was infested with *S. virginica* (8–40 first to second instars per row) three times during V6–R4 stage of growth (31–56 dap) and at R3 stage of growth (41 dap) with 40 adults. Larvae and adults were obtained from *S. virginica* larvae collected on sunflower and reared to adulthood at Yanásuy (Venado Tuerto, Santa Fe Province, Argentina). The second screenhouse at Fontezuela could not be infested due to a shortage of *S. virginica* livestock.

Damage was assessed as described previously at R3 to R6 stage of growth (52–87 dap) in the *R. nu* screenhouses and R6 stage of growth (101 dap) in the *S. virginica* screenhouse. Damage data were analyzed as described previously.

High-Dose Bioassays. Lines 19459-55 and 19487-35 were evaluated for high dose expression of TIC107 against *A. gemmatalis* and *P. includens* by using dilution bioassays of lyophilized leaf tissues incorporated into artificial diet. These two pests were selected because of their differing sensitivity to Bt and their occurrence in the United States where the study was conducted. Insect eggs were obtained from laboratory colonies at Monsanto, Union City, TN (*A. gemmatalis*) and University of Georgia, Tifton (*P. includens*), and incubated in 2.8-liter polypropylene boxes (Rubbermaid, Wooster, OH) lined with lightly moistened tissue and incubated in an environmental chamber (#I-35VL, Percival, Boone, IA) at 27 ± 1°C, 60 ± 10% RH, and a photoperiod of 0:24 (L:D) h until hatch. Leaf tissue samples collected for protein expression analysis as described above were used in this study. After taking a portion of the samples for ELISA, the remaining tissues were transferred to 50-ml plastic centrifuge tubes, weighed, and lyophilized overnight in a freeze dryer (VirTis, Gardiner, NY). Lyophilized samples were thawed and reweighed to establish fresh:dry weight correlations and pulverized by placing five to six 5/8-inch steel balls in each tube and shaking on a Harbil 5G-HD mixer for 3–6 min. Pulverized samples were stored at 4°C until used for bioassay.

Three bioassays were conducted against each species, with each including one replicate of each Bt line and one isoline at each plant growth stage from each location. Samples were warmed to room temperature and weighed into 50-ml plastic centrifuge tubes, using the fresh:dry weight correlation of each sample to calculate an amount that, when incorporated into artificial insect diet, would result in a 25-fold dilution relative to fresh tissue. Multiple species lepidopteran diet with mold inhibitor (Southland Products Inc., Lake Village, AR), modified by replacing the agar with analytical grade agar (#11393, Serva, Heidelberg, Germany) at a rate of 11.2 g/liter of diet, was prepared and cooled to ≈60°C in a water bath. Samples were incorporated by adding diet to a final volume of 17.5 ml each and agitating on a Vortex-Genie mixer (Scientific Industries, Bohemia, NY) at the highest setting until the diet was a uniform color, and no traces of sample were visible on the tube wall. Sample-laced diet was dispensed (0.5 ml/well) under a biological hood into 128-well bioassay trays (#BIO-BA-128, CD International Inc., Pitman, NJ) by using an Eppendorf Repeater 4780 (Brinkmann Instruments, Westbury, NY) and allowed to solidify. Treatments contained 28–32 wells, and two untreated controls (UTC) consisting of diet only (32 or 64 wells each) were included in each bioassay, one dispensed before sample incorporation and another afterward. A single *A. gemmatalis* or *P. includens* larva, 0–24 h posthatch, was transferred to each well with a fine camel's-hair brush. Trays were sealed with preventilated, self-adhesive covers (#BIO-CV-16, CD International Inc.) and incubated in an #I-35VL environmental chamber at 27 ± 1°C, 60 ± 10% RH, and a photoperiod of 0:24 (L:D) h. Bioassays were scored by recording the number of dead larvae and instar of survivors in each treatment after 11 d and correcting for untreated diet controls (Abbott 1925).

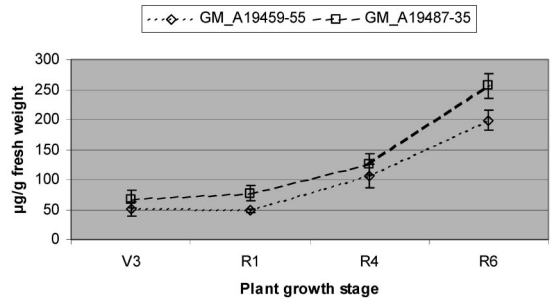

Fig. 1. Means and standard errors of expression of TIC107 (micrograms per gram fresh weight) in Bt soybean lines 19459-55 (diamonds, dotted line) and 19487-35 (squares, dashed line) at V3, R1, R4, and R6 stages of plant growth.

Results

Protein Expression. ANOVA determined that plant growth stage ($F = 51.67$, numerator [num] df = 3, denominator [den] df = 45, $P < 0.001$) and line ($F = 7.37$, num df = 1, den df = 45, $P = 0.0094$) were significant sources of variability in TIC107 expression, but location and replicate ($F = 0.02–0.18$, num df = 2, den df = 45, $P = 0.84–0.98$) were not. Average TIC107 expression approached or exceeded 50 μg/g fresh weight in both lines at V3 and R1 stages of growth and increased to >100 μg/g by R4 stage of growth (Fig. 1). By V6 stage of growth, average expression approached or exceeded 200 μg/g fresh weight, a significant increase over earlier growth stages ($F = 49.14$, num df = 3, den df = 50, $P < 0.0001$). Line 19487-35 exhibited slightly higher expression than line 19459-55, with the differences significant at R1 (t-value = 2.25, num df = 1, den df = 10, $P = 0.0483$) and R6 (t-value = 2.12, num df = 1, den df = 16, $P = 0.0496$) stages of plant growth.

2002 Argentina Screenhouse Trials. Because differences in larval incidence, larval damage, pod production, and yield among lines are caused primarily by differences in pest susceptibility, we compared lines for pests separately. In the *A. gemmatalis* trials, line was a significant source of variability in larval incidence ($F = 109.99$, num df = 2, den df = 24, $P < 0.0001$), damage ($F = 267.27$, num df = 2, den df = 24, $P < 0.0001$), pod production ($F = 61.86$, num df = 2, den df = 24, $P < 0.0001$), and yield ($F = 40.18$, num df = 2, den df = 9, $P = 0.0003$). Location was a significant source of variability in pod production ($F = 61.86$, num df = 1, den df = 24, $P < 0.0001$) but not larval incidence or damage ($F = 0.99–1.96$, num df = 1, den df = 24, $P = 0.18–0.33$). Replicate was not a significant source of variability for any parameter ($F =$

Table 1. Larval incidence (larvae per meter row [*A. gemmatalis*] or per 10 plants [*E. aporema*]), damage (percentage of defoliation [*A. gemmatalis*] or plants with damage [*E. aporema*]), pod yield (pods per plant), and seed yield (grams per 2-m row) in two Bt soybean lines compared with the parental ('A5547') line after infestation by *A. gemmatalis* or *E. aporema* in screenhouse trials at two locations in Argentina during the 2002 season

Line	Larval incidence	% damage	Pod yield	Seed yield
		A. gemmatalis (Fontezuela)		
A5547	451.1 ± 18.4a	96.3 ± 1.3a	4.8 ± 1.2b	
19478-8	1.3 ± 1.3b	1.0 ± 0.0b	57.0 ± 7.3a	
19487-35	0.4 ± 0.4b	1.0 ± 0.0b	61.6 ± 4.9a	
		A. gemmatalis (Oliveros)		
A5547	367.1 ± 74.5a	75.6 ± 6.3a	34.1 ± 2.9b	230 ± 27b
19478-8	3.4 ± 1.8b	4.7 ± 1.4b	97.2 ± 4.5a	713 ± 57a
19487-35	5.0 ± 4.0b	3.8 ± 0.5b	98.1 ± 8.7a	742 ± 42a
		E. aporema (Fontezuela)		
A5547	2.8 ± 0.9a	70.4 ± 5.0a	63.9 ± 15.9a	
19478-8	0 ± 0b	0 ± 0b	65.2 ± 5.3a	
19487-35	0 ± 0b	0 ± 0b	70.7 ± 5.8a	
		E. aporema (Oliveros)		
A5547	9.8 ± 1.3a	100 ± 0a	43.5 ± 4.2a	394 ± 77a
19478-8	0 ± 0b	0 ± 0b	62.6 ± 7.0a	439 ± 29a
19487-35	0 ± 0b	0 ± 0b	54.6 ± 3.5a	394 ± 34a

Values represent means ± SE. Means followed by the same letter within each column at each location do not differ significantly (Tukey–Kramer at $P = 0.05$).

Table 2. Larval damage in two Bt soybean lines compared with isogenic negative (neg) and parental ('A5547') lines after infestation by *A. gemmatalis* and *P. includens* in screenhouse trials at two locations in Illinois during the 2002 season

Line	% defoliation	
	A. gemmatalis	*P. includens*
	Jerseyville	
19459-55	0 ± 0c	0 ± 0b
19459-70 neg	63.8 ± 8a	73.8 ± 5.2a
19487-35	0 ± 0b	0 ± 0b
19487-31 neg	65 ± 8.7a	71.3 ± 7.5a
A5547	79.5 ± 1.9a	77.5 ± 0.8a
	Monmouth	
	A. gemmatalis	*P. includens*
19459-55	0 ± 0b	0 ± 0b
19459-70 neg	90 ± 5a	70 ± 4.6a
19487-35	0.5 ± 0.5b	0 ± 0b
19487-31 neg	93.8 ± 1.3a	71.3 ± 4.3a
A5547	93.5 ± 1.1a	73 ± 2.8a

Values represent means ± SE. Means followed by the same letter within each column at each location do not differ significantly (Tukey–Kramer at $P = 0.05$).

0.61–1.08, num df = 3, den df = 24 [9 for yield], P = 0.38–0.62). Incidence of *A. gemmatalis* larvae in the negative check was significantly greater than in the Bt lines at both location, and resulted in severe defoliation of negative checks but only trace damage to the Bt lines (Table 1). The slight damage to Bt lines observed at Oliveros is likely due to infiltration into the screenhouse by *S. frugiperda* larvae, which is relatively tolerant to Cry1A proteins (Nyouki et al. 1996), rather than feeding by *A. gemmatalis* larvae (J.C.G.). Increased larval incidence and damage in the negative check also resulted in significantly reduced pod production and yield (Table 1).

In the *E. aporema* trials, line was again a significant source of variability in larval incidence (F = 19.46, num df = 2, den df = 24, $P < 0.0001$) and damage (F = 234.02, num df = 2, den df = 24, $P < 0.0001$), but not pod production or yield (F = 0.22–1.16, num df = 2, den df = 24 [9 for yield], P = 0.34–0.81). Unlike the *A. gemmatalis* trials, location was a significant source of variability in *E. aporema* larval incidence (F = 6.10, num df = 1, den df = 24, P = 0.024), damage (F = 7.01, num df = 1, den df = 24, P = 0.017), and pod production (F = 4.58, num df = 1, den df = 24, P = 0.047). Replicate was again not a significant source of variability for any parameter (F = 0.11–1.97, num df = 3, den df = 24 [9 for yield], P = 0.16–0.95). No live larvae or damaged pods were encountered in either Bt line at either location (Table 1). Damage and live larvae were observed in the negative checks, but the levels were insufficient to reduce pod production or yield significantly.

2002 U.S. Screenhouse Trials. Line (F = 93.71, num df = 4, den df = 37, $P < 0.001$) and location (F = 21.75, num df = 1, den df = 37, $P < 0.001$) were significant sources of variability in *A. gemmatalis* damage, but replicate (F = 1.86, num df = 9, den df = 37, P = 0.09) was not. In the *P. includens* trial, line (F = 251.46, num df = 4, den df = 37, $P < 0.001$) was a significant source of variability in damage, but location (F = 1.42, num df = 1, den df = 37, P = 0.241) and replicate (F = 1.11, num df = 9, den df = 37, P = 0.31) were not. Both insects caused significant defoliation to negative checks at both locations, with that by *A. gemmatalis* approaching 100% at Monmouth (Table 2). Despite the severe pressure achieved with these insects at both locations, neither insect caused any discernable damage to either of the Bt lines at either location.

2002 U.S. Field Trials. Location (F = 10.07, num df = 4, den df = 114, $P < 0.001$), line (F = 17.61, num df = 4, den df = 114, $P < 0.001$), and treatment (F = 30.07, num df = 1, den df = 114, $P < 0.001$) were significant sources of variability in larval incidence, but replicate (F = 0.13, num df = 8, den df = 114, P = 0.998) was not. Location (F = 4.74, num df = 4, den df = 114, P = 0.001), line (F = 16.28, num df = 4, den df = 114, $P < 0.001$), and treatment (F = 67.90, num df = 1, den df = 114, $P < 0.0001$) were also significant sources of variability in larval damage, but again replicate (F = 0.07, num df = 8, den df = 114, P = 1.000) was not. Location (F = 203.64, num df = 4, den df = 114, $P < 0.001$), line (F = 3.37, num df = 4, den df = 114, P = 0.012), treatment (F = 24.44, num df = 1, den df = 114, $P < 0.001$), and replicate (F = 2.18, num df = 8, den df = 114, P = 0.034) were all significant sources of variability in yield.

Among lepidopterans, *P. includens* was the only pest encountered at Alexandria and the principle pest encountered at Leland and Loxley, whereas *A. gemmatalis* was the only pest encountered at St. Gabriel. Both species occurred at similar levels at Tifton. Other lepidopteran pests such as *H. zea; Playthpena scabra* (F.), green cloverworm; *Spodoptera exigua* (Hübner), beet

Table 3. Larval incidence, damage, and yield in two Bt soybean lines compared with isogenic negative (neg) or parental ('A5547') lines in sprayed versus unsprayed plots exposed to endemic lepidopteran populations at five locations in the southeastern United States during the 2002 season

Line	Larvae/m row (cumulative)		% defoliation (max)		Yield (kg/ha)	
	Unsprayed	Sprayed	Unsprayed	Sprayed	Unsprayed	Sprayed
			Alexandria			
19459-55	1.0 ± 0.3b$^{n.s.}$	1.0 ± 0.3b	0.0 ± 0.0b$^{n.s.}$	0.0 ± 0.0b	3,194 ± 33a$^{n.s.}$	3,278 ± 161a
19459-70 neg	81.5 ± 3.5a†	29.2 ± 3.9a	36.7 ± 3.3a†	10.0 ± 0.0a	3,294 ± 66a$^{n.s.}$	3,365 ± 169a
19487-35	1.0 ± 0.5b$^{n.s.}$	1.0 ± 0.6b	1.7 ± 1.7b$^{n.s.}$	0.0 ± 0.0b	2,846 ± 195a$^{n.s.}$	2,593 ± 350a
19487-31 neg	87.0 ± 6.1a†	24.5 ± 5.6a	40.0 ± 0.0a†	10.0 ± 0.0a	2,847 ± 300a$^{n.s.}$	3,108 ± 317a
			Leland			
19459-55	8.7 ± 3.4b$^{n.s.}$	4.0 ± 0.5b	0.0 ± 0.0b$^{n.s.}$	0.0 ± 0.0a	4,286 ± 238a$^{n.s.}$	4,098 ± 294a
19459-70 neg	260.1 ± 44.9a†	49.4 ± 10.3a	45.0 ± 5.0a†	0.7 ± 0.3a	3,904 ± 130a$^{n.s.}$	4,389 ± 219a
19487-35	9.5 ± 3.7b$^{n.s.}$	5.3 ± 1.1b	0.3 ± 0.3b$^{n.s.}$	0.0 ± 0.0a	4,063 ± 295a$^{n.s.}$	4,235 ± 249a
19487-31 neg	243.5 ± 15.9a†	56.1 ± 5.8a	53.3 ± 3.3a†	0.3 ± 0.3a	3,714 ± 112a$^{n.s.}$	4,374 ± 309a
			Loxley			
19459-55	1.3 ± 0.3b$^{n.s.}$	0.8 ± 0.3b	3.3 ± 0.7b$^{n.s.}$	1.3 ± 0.3b	2,784 ± 108a$^{n.s.}$	3,001 ± 72a
19459-70 neg	31.7 ± 7.8a†	11.9 ± 0.4a	26.7 ± 2.7a†	7.7 ± 0.3a	3,382 ± 79a$^{n.s.}$	3,457 ± 222a
19487-35	1.1 ± 0.6b$^{n.s.}$	0.3 ± 0.0b	2.7 ± 0.7b$^{n.s.}$	1.3 ± 0.3b	3,030 ± 217a$^{n.s.}$	3,046 ± 191a
19487-31 neg	25.2 ± 2.2a†	16.5 ± 4.6a	27.7 ± 1.5a†	7.3 ± 0.3a	3,239 ± 246a$^{n.s.}$	3,317 ± 167a
			St. Gabriel			
19459-55	1.2 ± 0.2b†	0.3 ± 0.2ab	0.0 ± 0.0b$^{n.s.}$	0.0 ± 0.0b	810 ± 131a†	1,226 ± 35a
19487-35	0.5 ± 0.0b†	0.0 ± 0.0b	0.0 ± 0.0b$^{n.s.}$	0.0 ± 0.0b	1,069 ± 191a$^{n.s.}$	1,481 ± 74a
A5547	52.4 ± 3.9a†	10.2 ± 2.3a	16.1 ± 0.7a†	5.4 ± 1.3a	479 ± 34b†	1,041 ± 111a
			Tifton			
19459-55	1.7 ± 1.5b$^{n.s.}$	0.5 ± 0.0b	0.0 ± 0.0b$^{n.s.}$	0.0 ± 0.0b	1,595 ± 111a$^{n.s.}$	1,837 ± 48a
19487-35	0.5 ± 0.0b$^{n.s.}$	0.2 ± 0.2b	0.2 ± 0.2b$^{n.s.}$	0.0 ± 0.0b	1,564 ± 101a†	1,857 ± 15a
A5547	39.2 ± 5.1a†	18.3 ± 1.3a	83.3 ± 3.6a†	8.1 ± 0.6a	1,432 ± 73a†	1,828 ± 57a

Values represent means ± SE. At each location, a separate ANOVA (Tukey–Kramer at $P = 0.05$) was conducted for treatments within each line (†, significant; n.s., not significant) and lines within each treatment (means followed by the same letter are not significantly different) for the three performance measurements.

armyworm; and *S. ornithogalli* (Guenée), yellow-striped armyworm were observed at one or more locations but at much lower levels compared with the principle pests. Larval incidence varied greatly among locations, with unsprayed negative checks exhibiting as little as 25.2 larvae per meter row (cumulative) at Loxley to as high as 260.1 larvae per meter row at Leland (Table 3). Regardless of infestation level, however, larval incidence in unsprayed plots of Bt lines was always significantly lower than in negative checks. The level of reduction in the Bt lines compared with negative checks ranged from 96 to 99%, with no significant differences noted between the two lines at any location. Damage also varied greatly among locations, with unsprayed negative checks experiencing the least damage at St. Gabriel (16.1% defoliation) and the most at Tifton (83.3% defoliation). Again, regardless of the amount of defoliation exhibited by unsprayed negative checks, no damage or only trace levels of damage were observed in the Bt lines at each location nor were any significant differences noted between the two lines.

Insecticide applications to sprayed plots succeeded in limiting defoliation in negative checks to 10% or less (Table 3). The degree to which larval incidence and damage were reduced compared with unsprayed negative checks varied by location, but the differences were significant in all cases. Despite this, larval incidence and damage remained significantly higher in sprayed negative checks than in sprayed plots of the Bt lines in all but one comparison (A5547 versus 19459-55 at St. Gabriel). The level of reduction in the sprayed Bt plots compared with sprayed negative checks ranged from 82 to 100%. Again, no significant differences in larval incidence or damage were detected between the two Bt lines, nor were any detected between sprayed and unsprayed plots of either Bt line.

Lepidopteran pest infestations were not sufficient to cause significant yield impacts at Alexandria, Leland, or Loxley (Table 3). At these three locations, no significant differences in yield were detected between lines within either treatment (sprayed or unsprayed), or between treatments within any of the lines. Significant yield differences were noted at Tifton. Yields in unsprayed plots of the negative check and both Bt lines were lower than sprayed plots, with the differences being significant for the negative check and one of the Bt lines (19487-35). No significant differences were detected among lines within the unsprayed or sprayed treatments. Significant yield differences were also noted at St. Gabriel, where harvest had to be delayed for several weeks due to rain. This had an overall negative impact on yield across all lines in both treatments. As observed at Tifton, yields in unsprayed plots of the negative check and both Bt lines were lower than sprayed plots, with the differences being significant for the negative check and Bt line 19459-55. Here, however, the unsprayed negative check yielded significantly less than unsprayed plots of the two Bt

Table 4. Larval damage in three Bt soybean lines compared with negative isogenic (neg) and parental ('A5547') lines after infestation by *R. nu* and *S. virginica* in screenhouse trials at one (*S. virginica*) or two (*R. nu*) locations in Argentina during the 2003 season

Line	% defoliation (season max)		
	R. nu (Fontezuela)	*R. nu* (Oliveros)	*S. virginica* (Oliveros)
19459-55	0.7 ± 0.5b	1.2 ± 0.4c	6.3 ± 1.4b
19478-8	0.7 ± 0.2b	1.7 ± 0.8c	8.5 ± 2.1b
19487-35	1.2 ± 0.7b	1.9 ± 0.8c	7.4 ± 1.2b
19459-70 neg	18.9 ± 3.9a	22.4 ± 1.4b	44.4 ± 1.6a
19478-8 neg	26.8 ± 6.9a	25.8 ± 2.9ab	42.8 ± 6.5a
19487-31 neg	23.1 ± 4.9a	26.5 ± 1.7ab	55.3 ± 5.1a
A5547	19.4 ± 3.9a	31.5 ± 3.3a	48.2 ± 4.1a

Values represent means ± SE. Means followed by the same letter within each column at each location do not differ significantly (Tukey–Kramer at $P = 0.05$).

Fig. 2. Percentage of mortality and stunting (not reaching third instar) of *P. includens* first instars after 11 d on artificial diet containing lyophilized leaf tissues of two Bt soybean lines and isogenic negative checks at a 25-fold dilution relative to fresh leaf tissues. Values represent means ± SE after corrected for untreated controls.

lines. No significant differences were detected among lines within sprayed plots.

2003 Argentina Screenhouse Trials. Line was a significant source of variability in *R. nu* larval damage ($F = 32.8299$, num df = 6, den df = 45, $P < 0.0001$) but not pod production ($F = 2.1961$, num df = 6, den df = 18, $P = 0.0915$). Neither location nor replicate were significant sources of variability in larval damage ($F = 3.0293$ and 0.6339, num df = 1 and 3, den df = 45, $P = 0.0886$ and 0.597), and replicate was not a significant source of variability in pod production ($F = 0.1111$, num df = 3, den df = 18, $P = 0.9525$). Only moderate pest pressure was achieved, with negative checks exhibiting defoliating levels of 32% or less (Table 4). None of the Bt lines, however, exhibited more than trace damage at either location. No significant differences in pod production were observed (data not shown).

In the *S. virginica* trial, line again was a significant source of variability in larval damage ($F = 36.2432$, df = 6, $P < 0.0001$) but not pod production ($F = 0.9388$, num df = 6, den df = 18, $P = 0.4918$). Replicate was not a significant source of variability for either larval damage ($F = 1.2029$, num df = 3, den df = 18, $P = 0.3371$) or pod production ($F = 0.9113$, num df = 3, den df = 18, $P = 0.4551$). Good insect pressure was achieved in the trial, and all four negative checks exhibited significantly higher defoliation than all three Bt lines (Table 4). As in the *R. nu* trials, no significant differences in pod production were observed among any of the lines (data not shown).

High-Dose Bioassays. The negative check from the R6 samples from Leland (collected one day after an insecticide application) caused 47% mortality of *P. includens* in the first bioassay. As a result, all R6 samples from Leland were excluded from the study. Additionally, sample quantities of one replicate each of 19459–55 at V3 and 19487–35 at R1 were limited, thus, another replicate of each was used in two bioassays against *A. gemmatalis.* In the *P. includens* bioassays, untreated control mortality ranged from 0 to 3.1% ($n = 3$). Diluted tissues of both Bt lines caused complete or near complete mortality after 11 d exposure (Fig. 2). In contrast, tissues from negative controls caused almost no mortality. In both Bt lines, what few survivors were present after 11 d were severely stunted relative to control larvae, with virtually none of them able to reach third instar. In the three successive *A. gemmatalis* replicates, untreated control mortality was 6%, 15%, and 25%, respectively. The reason for the relatively higher control mortality, especially in the third replicate, is unknown. Nevertheless, diluted tissues of both Bt lines caused complete mortality after 11 d exposure, whereas corrected mortality in negative controls did not exceed 7% (Fig. 3).

Discussion

These data suggest that expression of TIC107 in Bt soybean lines 19458-55, 19478-8, and 19487-35 all provide near complete control of a variety of lepidopteran soybean pests. It also seems that expression of TIC107 in these lines is more effective in reducing larval incidence of lepidopteran pests and their damage than prophylactic application of insecticides, because larval incidence and damage were nearly always lower in unsprayed plots of these lines than in sprayed negative

Fig. 3. Percentage of mortality and stunting (not reaching third instar) of *A. gemmatalis* first instars after 11 d on artificial diet containing lyophilized leaf tissues of two Bt soybean lines and isogenic negative checks at a 25-fold dilution relative to fresh leaf tissues. Values represent means ± SE after corrected for untreated controls.

check plots. Our analyses of protein expression in lines 19458-55 and 19487-35 also suggest that TIC107 expression at a given growth stage is consistent in multiple field environments.

Yield reductions in unsprayed negative checks were expected at Leland and Tifton due to the high amount of defoliation exhibited relative to sprayed checks. No such reductions were observed at Leland, possibly because the majority of damage occurred after plants had reached R6 stage of growth. Although the relationship between defoliation and yield in soybean is complex (Higley 1992), defoliation during R6 stage of growth generally has a lower impact on yield than during earlier growth stages (Turnipseed and Kogan 1976). Significant yield reductions did occur at Tifton, where much higher levels of lepidopteran damage were observed. Here too, however, most of this damage occurred after plants had reached R6 stage of growth, and the two Bt lines also exhibited similar yield reductions (significant for 19487-35) despite a near complete absence of lepidopterans or their damage in these lines. As a result, yield reductions at this location are likely a result of stink bugs, which reached elevated levels in unsprayed plots but were controlled in sprayed plots. Yield reductions were not expected at St. Gabriel due to a low overall incidence of lepidopterans, but reductions did occur and at similar levels in all three lines. This again is likely a result of stink bugs, which were also noted at this location.

The high efficacy exhibited by these Bt soybean lines against lepidopteran pests is undoubtedly due to very high TIC107 expression levels. TIC107 is nearly identical to Cry1Ac, differing by only seven amino acids in the highly conserved Domain I region of the active toxin (Fischhoff and Perlak 1995). It is identical to the Bt protein expressed in Bollgard transgenic cotton, which exhibits virtually complete efficacy against tobacco budworm, *Heliothis virescens* (F.), and pink bollworm, *Pectinophora gossypiella* (Saunders); and good suppression of *H. zea* (Wilson et al. 1992, Benedict et al. 1996, Jenkins et al. 1997, Henneberry and Jech 2000, Carrière et al. 2003). Expression levels in reproductive tissues of Bollgard averaged 57 μg/g dry weight ($\approx$8.6 μg/g in fresh tissues) at earlier plant growth stages but decreased $\approx$10-fold later in the season (Greenplate 1999). In contrast, Bt lines 19459-55 and 19487-35 exhibited much higher expression levels, averaging $\approx$50 μg/g fresh weight at V3 stage of growth and increasing to $\approx$200–250 μg/g fresh weight by R6 stage of growth. These values are 104–1,190 times higher than the range of LC_{50} values reported for purified Cry1Ac against three *P. includens* populations (Luttrell et al. 1999), 74–968 times higher than for those reported for four *E. aporema* populations (Gamundi et al. 2004), and 28–446 times higher than those reported for three *R. nu* populations (Gamundi et al. 2004). Although reports on the in vitro activity of purified Cry1Ac against *A. gemmatalis* are lacking, this insect has shown significantly greater susceptibility than *P. includens* to Bt spray formulations based on the HD1 strain of Bt subsp. *kurstaki* (Ignoffo et al. 1977, Morales et al. 1995). Thus, TIC107 expression in these lines presumably exceeds greatly the range of LC_{50} values expected for this species also.

These lines are apparently more efficacious than Jack-Bt transgenic soybean, which expresses a synthetic version of a truncated *cry1Ac* gene (Stewart et al. 1996) and exhibited partial efficacy against *A. gemmatalis*, *P. includens*, and *H. zea* in screenhouse trials (Walker et al. 2000). *P. includens* in particular caused significant damage to Jack-Bt soybean in one trial (>60% defoliation), and an economic injury threshold of 35% defoliation during vegetative stages of plant growth (Sullivan and Boethel 1994) was observed in at least one trial for each pest. This was attributed to migration of older larvae from negative checks to neighboring Bt soybean plots, on which they were able to feed due to their presumed greater Cry protein tolerance. We also observed larval migration in many of our screenhouse trials, which used intermixed single-row plots of Bt lines and negative checks. However, those larvae that were observed on Bt plants typically were already dead or exhibiting symptoms of Bt toxicity (e.g., lethargy, regurgitation of gut contents), and in no case were larvae of any age observed actively feeding on Bt plants. In trials where heavy insect pressure was achieved, we often observed large numbers of dead late instars on the ground under the Bt soybean plants on which they had fed after migrating from adjacent negative checks, yet in no case was damage to the Bt soybean plants detected.

In addition to the high degree of efficacy exhibited by the Bt soybean lines evaluated in this study, high-dose evaluations on two of the lines also demonstrated season-long high dose against *A. gemmatalis* and *P. includens*. The FIFRA Scientific Advisory Panel (1998, 2001) concluded that a transgenic crop cultivar can be considered to provide high dose if two of five recommended approaches indicated such. Dilution bioassays are one approach, which in this study demonstrated that leaf tissues from lines 19459-55 and 19487-35 express TIC107 at levels at least 25 times higher than needed to cause complete (*A. gemmatalis*) or near complete (*P. includens*) mortality of first instars. The few *P. includens* larvae that did survive were severely stunted developmentally and would likely die under field conditions due to increased vulnerability to biotic and abiotic mortality factors. Another approach recommended by the FIFRA Scientific Advisory Panel is demonstration of virtually complete efficacy against target pests in multiple field and screenhouse trials. The data provided herein support such a conclusion for lines 19459-55 and 19487-35 against both of these pests, as virtually no larval establishment or damage were observed on either line in any of the open field or screenhouse trials conducted. Thus, we propose that these lines express high dose against these two pests. Direct high dose evaluations remain to be conducted for line 19478-8; however, this line exhibited efficacy equivalent to lines 19459-55 and 19487-35 in all screenhouse and field trials conducted. Also remaining to be determined is the potential impact of deploying Bt soybean in areas where Bollgard

cotton is grown, because some lepidopteran species use both crops as hosts.

Lines 19459-55, 19478-8, and 19487-35 represent the first Bt soybean lines exhibiting high efficacy against multiple pests in multiple geographies. This efficacy was maintained throughout the growing season in all trials and across four plant generations (R_2–R_5), suggesting stable transgene expression within and across generations. In addition, transgene expression in the two lines evaluated represented a high dose against two of the most important lepidopteran pests in the United States and South America. The presence of a single, simple transgenic insert and absence of marker genes or additional DNA sequences outside of the T-DNA region also make these lines potentially suitable for commercial release after regulatory acceptance/approval. Confirmation of efficacy, yield performance, and other agronomic characters in additional soybean genotypes will be required to confirm the commercial suitability of these lines. Deployment of one of these lines has the potential to enable better pest management in soybean with fewer applications of chemical insecticides, creating economic and environmental benefits similar to those realized from the commercialization of Bt corn and Bt cotton.

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