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Characterization of Soybean Exhibiting High Expression of a Synthetic *Bacillus thuringiensis cry1A* Transgene That Confers a High Degree of Resistance to Lepidopteran Pests

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ABSTRACT

We report the generation of transgenic soybean [*Glycine max* (L.) Merr.] via *Agrobacterium*-mediated gene transfers of a *cry1A* gene (*tic107*) from *Bacillus thuringiensis* (*Bt*) that exhibits a high degree of resistance against the lepidopteran pests *Pseudoplusia includens* (Walker) (soybean looper), *Helicoverpa zea* (Boddie) (soybean podworm), and *Anticarsia gemmatalis* Hübner (velvetbean caterpillar). Three transgenic soybean lines (862, 726, and 781) were evaluated for expression, molecular composition, efficacy against target pests, and agronomic characteristics. We have designed and tested an expression cassette that consistently and reproducibly generates transgenic soybeans that accumulate the tic107 protein at levels as high as 6.12 μg mg^{-1} of total extractable protein. Expression levels of this magnitude of a *Bt* insecticidal protein have never been reported in soybean. Previous reports have indicated expression levels 100-fold lower in the highest-expressing lines. In addition, the phenotypes of these high-expressing lines were indistinguishable from their negative segregant and the transformation parent (Asgrow var. 'A3237'). Insect bioassay data demonstrate complete protection against soybean looper, soybean podworm, and velvetbean caterpillar when negative controls exhibited defoliation as high as 98%. Unlike previous reports of transgenic soybeans, we report here highly efficacious, single-copy, and normal phenotypes of transgenic soybean plants containing the highly expressed *cry1A* gene.

MANY OF THE ECONOMICALLY SIGNIFICANT insect pests of soybean are lepidopterans and are especially important in areas with long growing seasons such as the southeastern USA and South America (Higley and Boethel, 1994; Aragón et al., 1997). These insect species, primarily soybean looper, velvetbean caterpillar, and soybean podworm, are responsible for up to 44% of the economic insect damage in the southeastern USA, depending on the year (McPherson et al., 1999). Chemical insecticides are commonly used for controlling infestations of lepidopteran pests in soybean; however, narrow application windows, the emergence of insecticide resistance, and public pressure for reduced pesticide use limit the desirability of this approach to pest management (Aragón et al., 1997; Thomas and Boethel, 1994). Alternative control strategies utilizing biological insecticides are available but have not gained widespread acceptance (Luttrell et al., 1998; Moscardi, 1999). Recent advances in crop biotechnology offer transgenic plants as an efficient and environmentally friendly alternative strategy for controlling lepidopteron insects in regions where these pests are of agronomic concern (Williams et al., 1998).

Bacillus thuringiensis, a common soil bacterium, is found throughout the environment. For >50 yr *Bt* toxins have been used to control certain insect pests (Tabashnik et al., 1994). Expressed as crystal proteins, *Bt* δ-endotoxins are an effective way of controlling certain insects because their insecticidal activity is limited to certain insect orders. In addition, δ-endotoxins are highly specific and are nontoxic to mammals and other species (Betz et al., 2000), making them a safer and more environmentally benign alternative to synthetic chemical insecticides. *Bacillus thuringiensis* proteins (Cry proteins) are encoded by *cry* genes and synthesized as protoxins during bacterial sporulation, then solubilized and activated under alkaline conditions of the insect midgut. The insertion of the activated protein molecules into the cell membranes of the midgut epithelium results in formation of cation channels that lead to osmotic imbalance and lysis of the midgut cells, resulting in gut paralysis and, eventually, insect death (Knowles, 1994). Transgenic expression of *Bt* proteins has proven to be a highly effective method for controlling insect pests, and nearly all major crop plants, including maize (*Zea mays* L.), rice (*Oryza sativa* L.), cotton (*Gossypium hirsutum* L.), potato (*Solanum tuberosum* L.), tomato (*Lycopersicon esculentum* Mill.), tobacco (*Nicotiana tabacum* L.), soybean, and canola (*Brassica napus* var. *napus*) (Perlak et al., 1990, 1993; Stewart et al., 1996a; Armstrong et al., 1995; Parrott et al., 1994) have been engineered to express one or more of these proteins. This has resulted in enormous economic and environmental benefits as a result of increased yield with significant reductions in the use of chemical insecticides (Perlak et al., 2001; Shelton et al., 2002).

Much attention has been paid by both academics and industry experts in formulating strategies to prevent or delay the development of *Bt* resistance among insect pests (Tabashnik, 1989; Tabashnik et al., 1994; Huang et al., 2000; Andow et al., 2000; Lewis et al., 1997). One insect resistance management strategy currently used with *Bt* crops is so-called "high dose" (Gould, 1994) expression of the *Bt* protein combined with a refuge of

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Abbreviations: *Bt*, *Bacillus thuringiensis*; PCR, polymerase chain reaction; RT, room temperature; T-DNA, transfer DNA.

nontransgenic plants (FIFRA Scientific Advisory Panel, 1998). In this strategy, expression of the *Bt* protein is sufficient to kill not only susceptible genotypes, but also heterozygyous-resistant genotypes, the most common carriers of resistance alleles. This renders any resistance alleles in the pest population functionally recessive, and maintenance of susceptible insect genotypes by the refuge prevents interbreeding among the rare homozygous-resistant insect genotypes. To date, there have been limited reports of transgenic soybean (Stewart et al., 1996b; Walker et al., 2000; Dufourmantel et al., 2005); however, their expression levels and corresponding efficacy have not been sufficient to qualify as high dose against any of the primary lepidopteran pests of soybean. We report here an expression cassette that results in greatly increased expression of a Cry1Ac-like *Bt* protein in soybean plants. These plants exhibit a high degree of resistance to lepidopteran insects with no apparent negative phenotypic effects. Data are also provided on the genetic integrity of the transgene insert.

MATERIALS AND METHODS

Plasmid Construction

The synthetic *tic107*, patterned after the *Bt* ssp. *kurstaki cry1A*c sequence, was designed for high expression in plants as described by methods published in the patent by Fischhoff and Perlak (1996). The synthetic *tic107* nucleotide sequence found in this patent is referred to as synthetic B.t.k. HD-73 (also known as Cry1Ac). We renamed this protein tic107 because the encoded mature protein produced by this gene is not identical (99.4%) to the naturally occurring Cry1Ac protein produced by *B. thuringiensis* subsp. *kurstaki.* The *Bt* expression cassette was assembled in a high-copy pUC vector and inserted into a binary vector via a Not I site (Fig. 1). The binary vector contained a DNA fragment from the pTiT37 plasmid containing the 24 bp nopaline-type transfer DNA (T-DNA) right border used to initiate the T-DNA transfer from *Agrobacterium tumefaciens* to the plant genome (Depicker et al., 1982; Bevan et al., 1983) and octopine left border from octopine Ti plasmid TiA6 (Baker, 1989). Promoter from *A. tumefaciens* pTiT37 for the gene encoding nopaline synthetase (Bevan et al., 1983) was responsible for the expression of the gene isolated from Tn5 (Beck et al., 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley et al., 1983). The 3′ nontranslated region of the nopaline synthetase gene from *A. tumefaciens* was used to terminate transcription and direct polyadenylation of the nptII mRNA (Depicker et al., 1982; Bevan et al., 1983). Plasmid PV-GMBT01, contained the origin of replication derived from the broad-host plasmid RK2 (Stalker et al., 1981). The gene for 3″ (9)-O-aminoglycoside adenylytransferase allowed for bacterial selection on spectinomycin or streptomycin (Fling et al., 1985).

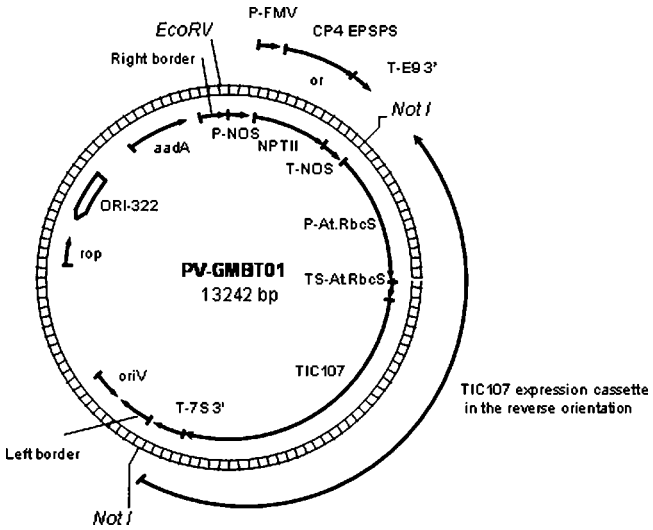

Fig. 1. Plasmid map of PV-GMBT01. *Not I* fragment contains the *Bt.* expression cassette. ori = microbial origin of replication. Either CP4 EPSPS (5-enolpyruvulshikimate-3-phosphate synthase [EPSPS] sequence isolated from *Agrobacterium* sp. strain CP4) or NPTII (neomycin phosphotransferase) was the selectable marker used in plant transformation. Spectinomycin or streptomycin resistance was used as a bacterial marker (*aadA*). Restriction enzyme sites for *Eco RV* and *Not I* are shown.

Transformation and Segregation Analysis

Soybean plants expressing the Cry1A-like δ-endotoxin (Adang et al., 1985) from *B. thuringiensis* var. *kurstaki* were generated in 1996. The disarmed *A. tumefaciens* plant transformation system was used to produce all soybean lines as described in the Hinchee and Connor-Ward (1995) patent. This delivery system is well documented to transfer and stably integrate T-DNA into a plant nuclear chromosome (Klee et al., 1983). Vector PV-GMBT01 was mobilized into disarmed *A. tumefaciens* strain ABI and selected on spectinomycin and chloramphenicol (Fig. 1). Soybean cotyledons were used as explant sources and were infected with the *Agrobacterium* culture. Following co-culture, *Agrobacterium* were killed using a culture media containing the appropriate antibiotics. Explants were later placed on kanamycin or glyphosate selection medium. Developing shoots were excised from an R_0 plant, positive shoots were grown to maturity, selfed to produce seed, and their progeny were screened for the expression of *Bt*. The presence of the gene was established by detecting the *Bt* protein using B.t.k Corn genechecks (SD70755, Strategic Diagnostics, Inc., Newark, DE) according to the manufacturer's protocol.

Agronomic Testing

The trials were planted at three locations throughout Illinois. The experimental design was a split plot design with positive and negative isolines for each event paired within each of the four replications. The main plot represented the event and the split plot represented the presence or absence of tic107. A nontransgenic varietal control, A3237, was included, unpaired, at the same frequency as each pair of transgenics. Each replication consisted of three paired (pos and neg) transgenic events per event and three unpaired A3237 plots. The plots were four rows wide by 5.18 m (17 ft) long. The seeding density was 136 seeds row^{-1} (≈8 seed ft^{-1}) and the row spacing was 76.2 cm (30 in). A commercial soil residual herbicide was applied to the entire experimental area to maintain weed-free plots.

Statistical analysis of data was done using JMP software (SAS Institute Inc., Cary, NC, USA). Each pair of means was compared by one-way ANOVA using the Student's test. A P value of <0.05 was considered statistically significant. Emergence, flowering, plant height, lodging, maturity, and yield were measured within each line by each treatment compared with the untreated check across locations.

Molecular Characterization

Genomic DNA was isolated from R_1, R_2, R_3, and R_5 generation tissue from plants of A3227 (nontransformed) and each transgenic line grown under greenhouse conditions (14/10 h day/night photoperiod at 200 μmol photons m^{-2} s^{-1}), with daytime temperatures of 32°C and nighttime temperatures of 22°C. Genomic DNA was extracted from ≈1 g of young trifoliolate leaf tissue using a Nucleon Plant DNA extraction kit (RPN8511, Amersham Pharmacia Biotech, Piscataway, NJ). After the final precipitation step, DNA was resuspended in 0.5 mL of $T_{10}E_1$ and quantified using DyNA Quant 200 Flourometer (Amersham Pharmacia Biotech, Piscataway, NJ). Purified genomic DNA was digested with various restriction enzymes, run on a 0.8% agarose TAE gel for ≈16 h at 45 V, transferred to a zeta-probe GT nylon membrane (162-0197, BioRad, Hercules, CA), and DNA was crosslinked to the nylon membrane. Templates for radioactive probe synthesis were prepared from gel-purified restriction fragments of PV-GMBT01 or generated by polymerase chain reaction (PCR) using oligonucleotides that anneal at the ends of the respective element. Probes were labeled using the Klenow fragment of DNA polymerase I, random oligomers, and ^{32}P-dCTP (GIBCO BRL RadPrime DNA labeling system, Invitrogen, Carlsbad, CA). After hybridization, blots were washed at increasing stringency with the final wash being 0.1 × SSC (1 × SSC, 150 m*M* sodium chloride, 15 m*M* sodium citrate, pH 7.0), 0.5% sodium dodecyl sulfate at 68°C. Multiple exposures of the blot were generated using Kodak Biomax MR film in conjunction with a Kodak Biomax MS intensifying screen (Eastman Kodak, Rochester, NY) including manyfold overexposure to permit detection of faint signals.

Detection of tic107 by Western Blotting and ELISA

Tissue Collection

Plants were grown under growth chamber settings as follows: 14/10 h (day/night) photoperiod at 200 μmol photons m^{-2} s^{-1}, with a daytime temperature of 32°C and nighttime temperature of 22°C. The humidity was set at 50%. There were two irrigation cycles per day, one with nutrients and water for 2 min and a second one with water for 1 min. The center leaflet of the newest fully expanded trifoliolate was folded, and an open 1.5-mL MicroTube (Sarstedt Inc., Newton, NC) was snapped closed over the leaflet, producing two leaf disks in the tube. The leaf tissue was stored on dry ice during collection of subsequent samples and transferred to −80°C until removed from storage for extraction procedures.

Western Analysis

Total protein was extracted from the leaf discs by adding 800 μL of 1 × phosphate buffered saline, pH 7.4 (1666 789, Boehringer Mannhein, Indianapolis, IN) plus 0.05% Tween 20 (P9416, Sigma Chemical Co., St Louis, MO) (PBST) and using a tissue homogenizer (903475, Wheaton Science Products, Millville, NJ) to mechanically lyse the cells. Extracts were centrifuged at maximum speed (14 000 rpm) for 5 min, and an aliquot of the supernatant was removed and treated for 1 h at 37°C with trypsin at a concentration of 175 μg mL^{-1}. The reaction was stopped by adding 1.25 m*M* phenylmethylsulfonyl fluoride (PMSF). The above protein extractions were separated under denaturing conditions on a 4 to 20% polyacrylamide gel (OG-0420A, Owl Separation Systems, Portmouth, NH). Proteins were transferred to polyvinylidene fluoride (PVDF) membrane (IPVH20200, Millipore, Bedford, MA) via a semidry transfer system. The membrane was blocked to prevent nonspecific binding with NET/0.25% gelatin for 1 h at room temperature (RT). Primary antibody (B-3, rabbit anti-Cry1Ac1) was incubated at a 1:500 dilution in 1 × NET for 1 h at RT, and washed three times for 5 min with 1 × NET changing buffer after each wash. Secondary antibody (Anti-IgG-HRP) was incubated at a 1:5000 dilution in 1 × NET for 1 h at RT and washed as described above. Immunoreactive bands were visualized on x-ray film (RPN2103K, Amersham, Piscataway, NJ) using the Enhanced Chemiluminescent Kit (RPN2108, Amersham, Piscataway, NJ) according to manufacturer's instructions.

ELISA Assay

Five plants per event were sampled at 10, 14, 18, 22, 24, 30, 40, and 50 d after planting as described above. Each sample was evaluated in triplicate by ELISA and then averaged for one value per data point. Growth stage was recorded at time of sampling. For tic107 expression analysis, frozen leaf discs were combined with chrome-steel beads (BioSpec Products Inc., Bartlesville, OK), and extraction buffer (50 m*M* sodium carbonate/bicarbonate, 2 m*M* dithiothreitol, 0.07% Tween-20) was added at a 1:60 ratio (tissue wt.–vol.), then vigorously shaken in a Harbil Mixer 5G-HD (Fluid Management, Wheeling, IL) for 3.5 min. The extracts were placed on wet ice for 30 min to allow solid phase to settle, and an aliquot of supernatant was transferred to a new microcentrifuge tube. Trypsin was added to a final concentration of 50 m*M*. All samples were diluted 1:83 (for 1:15000 total dilution wt.–vol.) with TBA (100m*M* Tris base, 100 m*M* $Na_2B_4O_7$, 5m*M* $MgCl_2$, 0.05% Tween20, HCl to adjust pH to 7.8, and 0.2% L-ascorbic acid dissolved just before use) before loading on assay plates.

tic107 protein levels were determined by ELISA as described. Toxin significantly equivalent to the Cry1Ac encoded by *B. thuringiensis* subsp. *kurstaki* strain HD-73 expressed and purified from *Escherichia coli* fermentation was used as a standard. Lyophilized toxin was dissolved in 50 m*M* sodium bicarbonate (pH 9.6) and subjected to trypsinization as described for sample treatment. The standard was serially diluted in Tris-borate-Ascorbic Acid (TBA) to eight concentrations (32–0.25 ng mL^{-1}) to generate standard curve for quantitation of the expressed tic107. For ELISA assay, the 96-well Nunc immuno plates (MaxiSorp Nunc-Immuno plates, Nalge Nunc International, Roskilde, Denmark) were coated with a 1:48 000 diluted monoclonal IgG Ab1 (M19N4A6) and incubated overnight at 4°C. Plates were washed three times with PBST (0.001M KH_2PO_4, 0.01M Na_2HPO_4, 0.137M NaCl, 0.0027M KCl, 0.07% Tween20, pH 7.4), and blocked with PBST0 (1 × PBST, 0.5% oval albumin) at RT for 2 h. Plates were washed one time with PBST, loaded with 100 μL per well of freshly trypsinized sample [3 replicates $plate^{-1}$ for standards and samples, 6 $plate^{-1}$ for controls, and 8 $plate^{-1}$ for blanks (TBA)], and incubated at RT for 2 h. After three washes with PBST, plates were loaded with HD-73 polyclonal antibody diluted 1:1000 with PBST0 and incubated at RT for 1.5 h. Plates were washed three times with PBST, the bound polyclonal IgG were detected with donkey antirabbit IgG-HRP conjugate (Amersham, Piscataway, NJ) diluted 1:3000 with PBST0. After a final series of washes adding TMB peroxidase substrate, and developing for 10 min, plates were read for enzyme activities. Unknown optical density values were adjusted for background affects by subtracting mean nontransgenic (A3237) optimal densities. Unknowns were estimated from a standard curve generated by plotting the standard concentrations vs. the obtained mean optical density values.

Protein Assay

Total protein content of test soybean sample extracts were measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, New York), following the manufactors protocol for microtiter plates. Nontrypsinized extracts were diluted 1:10 with PBST (1:600 total dilution wt.–vol.) before protein assay. Bovine serum albumin 2.0 mg mL^{-1} standard (Pierce, Rockford, IL) was used to generate a standard curve. Six standard dilutions were prepared from 0.5 to 0.05 mg mL^{-1} using a dilution buffer similar to the unknown's 1:10 extraction buffer to PBST. Plates (96-well MaxiSorp Nunc-Immuno plates, Nalge Nunc International, Roskilde, Denmark) were read at 595 nm in Spectra Max plate reader with SOFTmax PRO 3.0 software (Molecular Devices Corporation, Sunnyvale, CA). Total protein levels were used to normalize tic107 protein amounts.

Insect Bioassays

Leaf Disc Bioassay

Two tissue types (newly expanded leaves and N4 leaves) were sampled from R_4 generation transgenic (862, 726, and 781) and isogenic plants at R_3 (862) or R_4 (726 and 781) stage of growth and evaluated against first-instar larvae of velvetbean caterpillar, soybean podworm, and soybean looper. Leaf discs were placed singly in wells of 24-well tissue culture plates containing 1.0 mL of 2% agar, infested singly with neonate larvae of the test insects (24 $treatment^{-1}$), and incubated for 4 d at 25°C under a 14L:10D photoperiod. Insect mortality was recorded and percent leaf consumption was estimated for each disc.

Whole Leaf Bioassay

Leaf feeding studies were conducted using third- and fourth-instar velvetbean caterpillar larvae obtained from insect rearing at Embrapa Soja (Londrina, Paraná, Brazil). Bioassays consisted of 60 larvae $treatment^{-1}$ (three replicates) including transgenic *Bt* lines 862, 726, and 781, and a *Bt* (negative) commercial check (M-SOY5826), conducted in the MONSOY Laboratory (Ponta Grossa, Paraná, Brazil). Larvae were fed with foliage in plastic boxes containing filter paper, with foliage being replaced as necessary. Larval mortality was recorded daily. Foliage consumption was measured through a portable, integrated leaf meter (LI-COR Model 3050A), estimating the foliage area offered to the larvae, the area remaining after feeding, and dividing the foliage area consumed by the number of larvae in each replicate. In the case where larvae grew in size and consumed heavily, the larvae in each replicate (principally the checks), were subdivided to fewer larvae in plastic boxes.

RESULTS

Plant Transformation

As plantlets regenerated and rooted, they were moved to soil and transferred to the greenhouse. One hundred and seven resistant events were produced that were regenerated into 87 plantlets for further testing under greenhouse conditions. Once in the greenhouse, plants were assayed for expression of *Bt* at approximately the V3 to V4 leaf stage. Lines were advanced, based on expression of *Bt* that behaved as a single dominant gene inherited in a Mendelian fashion (fit a 3:1 segregation ratio) at R_1. Seven lines were moved forward for molecular characterization (851, 859, 862, 863, 726, 781, and 1085) and single copy events were advanced (862, 726, and 781). All events contained the selectable marker nptII with the exception of event 781 which contained CP4. The same phenotypic test was done at R_2 and R_3 to determine homozygous individuals by identifying R_2 individuals whose progeny were all positive for expression of *Bt* (Table 2).

Characterization of Transgene

Extensive restriction enzyme mapping was done to evaluate copy number and integrity of transgenic elements on seven transgenic *Bt* lines. The results from genomic DNA digest with five single enzymes and two enzyme combinations confirmed the copy number and integrity of the integrated transgene in each line (data not shown). Figure 2 is a Southern blot probed with the entire transformation plasmid showing the transgene copy number results, as determined by digesting genomic DNA with a restriction enzyme that cleaves once within the T-DNA (*EcoR V*). This restriction enzyme and probe combination should produce a single unique band if there is a single copy of the T-DNA inserted in the genome of the transgenic soybean line. Lines 851,

Table 1. Mean agronomic data compared with isogenic and parental line with no significant differences at the 95% confidence level.

Location	Line	Emergence (1–9)†	Flowering‡	Height§	Lodging (1–9)¶	Maturity#	Yield††
			DAP	cm		d	Mg ha^{-1}
Wyoming, IL	862 (Iso)	1.0 (1.0)	41.8 (41.5)	76.2 (76.2)	2.0 (2.0)	117 (117)	2.38 (2.44)
	726 (Iso)	1.0 (1.0)	35.3 (34.8)	76.2 (76.2)	2.0 (2.0)	117 (117)	2.40 (2.63)
	781 (Iso)	1.0 (1.0)	35.5 (34.3)	76.2 (76.2)	2.0 (2.0)	117 (117)	2.45 (2.55)
	A3237	1.0	34.0	76.2	2.00	117	2.74
Monmouth, IL	862 (Iso)	1.0 (1.0)	41.3 (41.5)	83.6 (87.1)	1.0 (1.0)	115 (115)	3.15 (3.25)
	726 (Iso)	1.0 (1.0)	37.5 (37.8)	87.1 (88.6)	1.0 (1.0)	115 (115)	3.08 (3.34)
	781 (Iso)	1.0 (1.0)	38.3 (37.8)	88.9 (86.4)	1.0 (1.0)	115 (115)	3.27 (3.21)
	A3237	1.0	39.3	86.1	1.0	115	3.53
Carlyle, IL	862 (Iso)	1.0 (1.0)	34.0 (34.3)	69.3 (71.9)	1.0 (1.0)	90 (90)	2.75 (2.70)
	726 (Iso)	1.0 (1.0)	34.3 (34.3)	74.9 (77.5)	1.0 (1.0)	97 (97)	3.10 (3.32)
	781 (Iso)	1.0 (1.0)	34.0 (4.3)	78.7 (73.2)	1.0 (1.0)	97 (97)	3.20 (3.45)
	A3237	1.0	34.3	73.7	1.0	97	3.18

† Emergence for each plot was rated when nearly all the seedlings had emerged (1 is excellent; 9 is poor).
‡ Days after planting (DAP) when first open flower was observed in each plot.
§ Height: Plant height was recorded at maturity in centimeter. An average of three plants per plot was recorded.
¶ Lodging: Lodging was recorded at harvest (1 = straight up; 9 = completely down).
# Maturity: Days from planting when 95% of the pods were brown.
†† Yield adjusted for moisture content.

Table 2. Segregation data and analysis of progeny for *Bt* expressing lines.

Line	Generation tested	Observed†		Expected		χ^2
		Positive	Negative	Positive	Negative	
862	R_1	22	7	21.75	7.25	0.01ns‡
726		20	7	20.25	6.75	0.01ns
781		12	6	13.50	4.50	0.67ns
		homozygous at R_1	heterozygous at R_1	homozygous at R_1	heterozygous at R_1	
862	R_2	3	9	3.00	9.00	0.00ns
726		14	25	9.75	29.25	2.47ns
781		3	10	3.25	9.75	0.03ns

† R_1 data expressed as number of positive and negative plants based on presence of *Bt* detected by gencheck. R_2 data expressed as number of homozygous and heterozygous R_3 plants. Zygosity of R_1 plants were established when all R_2 progeny tested (15 plants) were either all positive for *Bt* (homozygous) or segregating for *Bt* (heterozygous).
‡ ns, not significant at $P = 0.05$ ($\chi^2 = 3.84$, 1 df).

859, and 863 contained multiple integration sites of T-DNA as demonstrated by multiple bands in Fig. 2. Line 1085 exhibited a single band when digested with EcoR V, which is indicative of a single integration site; however, digestion with multiple enzyme revealed an additional copy of the transgene that co-migrated (Fig. 2, Lane 10). Lines 863 and 1085 contain two intact copies of the T-DNA. Line 851 contains one intact and one truncated copy of *tic107*. The truncated insert was confirmed by PCR and sequence analysis, which showed the *nptII* gene was also truncated and the 5′ *tic107* fragment was in reverse orientation (data not shown). Line 859 contains five fragments of *nptII*, some of which were linked (data not shown). All other lines containing more than one inserted T-DNA were linked. Further evaluations of transgenic *Bt* lines were limited to lines 862, 726, and 781, which contained a single intact transgene. The three single copy lines were also evaluated by Southern blot for transgene stability at R_1, R_3, and R_5 (line 726 was not evaluated at R_3) and were stably integrated at a single locus (Fig. 3).

Protein Expression

Five plants were averaged together for one data point used in Table 3. tic107 expression levels were quantified to make relative comparisons between *Bt* expressing lines. Early-season expression levels of tic107 in all transgenic events were not significantly different from V1 through V3 with the exception of 862, which was significantly less than 726 at V2 ($P < 0.05$). However, line 781 exhibited late-season expression of tic107 significantly lower at V5 and V7 ($P < 0.05$) and at first bud through full pod ($P < 0.001$). During pod set, lines 862, 726, and 781 all expressed tic107 at significantly different levels ($P < 0.001$), with means of 2.68 ± 0.20, 1.26 ± 0.10, and 0.52 ± 0.09, respectively (Table 3). All lines exhibited relatively stable expression levels of tic107 expression over the different plant developmental stages and expressed the expected 64 kDa protein that was immunoreactive to antibodies raised to Cry1Ac. In addition, the 64 kDa protein absent in isoline 862 is present in the transgenic line 862 as shown in Fig. 4,

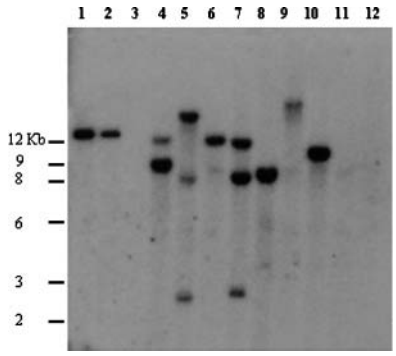

Fig. 2. Southern blot analysis of R_2 transgeneic and control soybean lines to identify copy number. Each lane represents plasmid DNA or 7ug genomic DNA. DNA was digested with EcoRV and subjected to electrophoresis in a 0.8% agarose gel then transferred to a nylon membrane, hybridized to a ^{32}P-labeled probe with PV-GMBT01, and subjected to autoradiography. Each band represents inserted DNA. The soybean line A3237 was the recurrent parent in all material examined: (1) 100pg PV-GMBT01, (2) 50pg PV-GMT01, (3) blank, (4) 851, (5) 859, (6) 862, (7) 863, (8) 726, (9) 781, (10) 1085, (11) 781 isoline, and (12) A3237 recurrent parent (negative control).

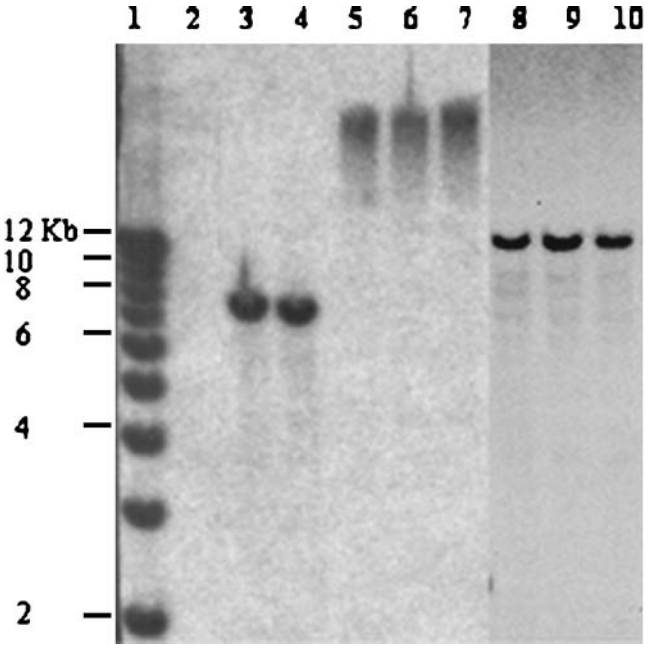

Fig. 3. Transgene stability of *Bt* events. Soy genomic DNA (10 μg) was digested with *Eco*RV, electrophoresed in a 0.8% agarose gel, transferred to a nylon membrane, hybridized to a ^{32}P-labeled probe with PV-GMBT01, and subjected to autoradiography. Individual lanes: (1) Molecular weight markers, (2) A3237 nontransgenic control, (3) 726 R_1, (4) 726 R_5, (5) 781 R_1, (6) 781 R_3, (7) 781 R_5, (8) 862 R_1, (9) 862 R_3, and (10) 862 R_5. Molecular weight markers are indicated on the side of the autoradiogram.

Table 3. Mean values of *Bt* protein detected in leaf tissue measured by ELISA. Values represent means ± SE.

Line	V1 (10 DAP)†	V2 (14 DAP)	V3 (18 DAP)	V5 (22 DAP)	V7 (26 DAP)	First bud (30 DAP)	Pod set (40 DAP)	Full pod (50 DAP)
	μg mg^{-1} of total extractible protein							
862	ND‡	2.56 ± 0.21§	ND	4.06 ± 0.83	ND	4.13 ± 0.41	2.68 ± 0.20	2.74 ± 0.25
726	4.66 ± 0.51	6.12 ± 0.89	3.34 ± 0.59	4.41 ± 1.06	4.37 ± 0.68	3.64 ± 0.58	1.26 ± 0.10	2.77 ± 0.89
781	3.60 ± 0.60	4.79 ± 1.20	1.73 ± 0.93	1.92 ± 0.29*	1.25 ± 0.31*	0.69 ± 0.18**	0.52 ± 0.09**	0.77 ± 0.36**

* Indicates probability of transgenic means being significantly different from other transgenic lines, $P < 0.05$.
** Indicates probability of transgenic means being significantly different from other transgenic lines, $P < 0.01$.
† DAP = days after planting.
‡ ND = data not taken.
§ P value indicates probability of transgenic means being significantly different from event 726, $P < 0.05$.

Lanes 1 and 5. This band was also absent in the parental variety A3237 (Fig. 4, Lane 4).

Insect Bioassays

Velvetbean Caterpillar

Both young (center leaflet of newest fully expanded trifoliolate) and old leaves (N4 leaves) of all three transgenic *Bt* lines caused 100% mortality in the 4-d test period, while mortality on the isogenic lines ranged from 25 to 42%. There were no apparent differences in mortality between isolines or tissue types. No tissue consumption was observed for either tissue type in any of the transgenic *Bt* lines, while isogenic lines exhibited 33 to 74% tissue consumption. There were no apparent differences in tissue consumption between isolines, however, for all three lines; young leaves were more heavily consumed than old leaves (Table 4). In the whole leaf bioassay, both third- and fourth-instar larvae on all three *Bt* soybean lines exhibited 100% mortality after 4 d, compared with 6.7 and 0% mortality, respectively, on the control (Tables 5 and 6). Third-instar larvae consumed an average 123 cm^2 of control leaf area, compared with only 0.1 to 0.2 cm^2 for the *Bt* lines (Tables 5 and 6). This corresponds to a reduction in feeding of virtually 100%. Figure 5 shows the inhibition of feeding by third-instar larvae caused by line 781, compared with the control, ≈24 h after initiation. Fourth-instar larvae-consumed an average of 117 cm^2 of control leaf area, compared with 0.5-0.6 cm^2 of the *Bt* lines, again representing a reduction in feeding of virtually 100%.

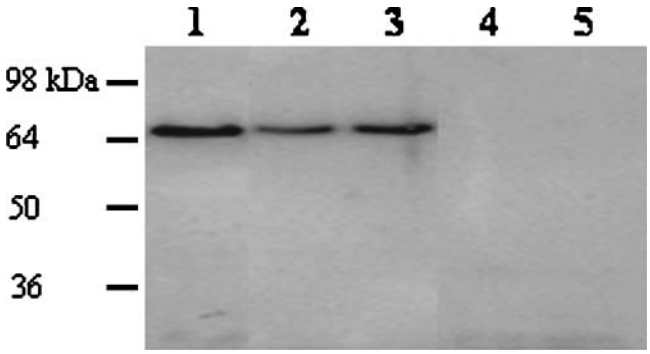

Fig. 4. Western Blot analysis of R_5 transgenic and nontransgenic soybean lines. Total protein was extracted from leaves and digested with trypsin. Denatured protein was separated by sodium dodecyl sulfate–polyacrylamide gel electroporesis, blotted, and visualized using Cry1Ac antiserum and chemiluminescent detection system. The soybean line A3237 was the recurrent parent in all material examined: (1) 862, (2) 726, (3) 781, (4) A3237 soybean genotype (negative control), (5) negative segregent for line 862.

Soybean Podworm

Both young and old leaves of all three transgenic *Bt* lines caused 100% mortality in the 4-d test period, while mortality on the isogenic lines ranged from 0 to 17%. No tissue consumption was observed for either tissue type in any of the transgenic *Bt* lines, while isogenic lines exhibited 72 to 98% tissue consumption. In this case, there were no apparent differences in tissue consumption between isolines, nor were young leaves more heavily consumed than old leaves (Table 4).

Soybean Looper

Both young and old leaves of all three transgenic *Bt* lines caused 100% mortality in the 4-d test period, while mortality on the isogenic lines ranged from 0 to 5%. No tissue consumption was observed for either tissue type in any of the transgenic *Bt* lines, while isogenic lines exhibited 46 to 85% tissue consumption. Again, there were no apparent differences in tissue consumption between isolines, nor were young leaves more heavily consumed than old leaves (Table 4).

Agronomic Testing

There was no significant differences in emergence, flowering, plant height, lodging, maturity, and yield as

Table 4. Antibiosis trials using first-instar (soybean podworm, soybean looper, and velvetbean caterpillar) with tic107 expressing transgenic *Bt* vs. a segregant without the transgene (Iso).

Test Insect	Line	Leaf tissue type	% Mortality		% Defoliation	
			Bt	Iso	*Bt*	Iso
Soybean podworm	862	N4†	100	0	1	98
		new	100	4	2	74
	726	N4	100	0	1	83
		new	100	17	1	72
	781	N4	100	0	0	79
		new	100	0	1	97
Soybean looper	862	N4	100	5	0	73
		new	100	4	0	49
	726	N4	100	0	0	58
		new	100	0	0	62
	781t	N4	100	0	0	46
		new	100	4	0	85
Velvetbean caterpillar	862	N4	100	29	0	41
		new	100	38	0	61
	726	N4	100	39	0	33
		new	100	42	0	55
	781t	N4	100	25	0	48
		new	100	25	0	74

† N4 = node four leaf.

Table 5. Mortality (number and percentage) of third-instar velvetbean caterpillar larvae fed with transgenic *Bt* lines (862, 726, and 781) on different days after treatment (DAT) and mean foliage consumption per larva, compared with check (M-SOY 5826 is a commercial check). Bioassay I, Ponta Grossa, MONSOY Experimental Station.

	Mortality (No. larvae)						
Genotype	1 DAT	2 DAT	3 DAT	4 DAT	Total	Mortality	Mean foliage per larva
						%	cm^2
M-SOY5826	1	1	2	0	4	6.7	123.3
862	12	19	29	0	60	100	0.1
726	16	16	28	0	60	100	0.1
781	20	7	33	0	60	100	0.2

described in Table 1. There was no significant difference between the positive and negative isoline for each event. In addition, there was no significant difference between events.

DISCUSSION

Design of Expression Cassette

The *tic107* gene is under the control of the *Arabidopsis* ribulose 1,5 bisphosphate carboxylase small subunit (rbcS) 1A gene promoter (P-ArabSSU1A) (Krebbers et al., 1988). Plant promoters were considered due to the diminishing transcription levels of viral promoters during plant development and the need for expression in selective tissues where feeding occurs. In *Arabidopsis* and other higher plants, rbcS mRNA are encoded by a multigene family and their expression patterns can differ both quantitatively and qualitatively in response to light and development and in different tissues (Dedonder et al., 1993). Cheng et al. (1998) have shown rbcS mRNA exhibit diurnal patterns of expression, with peak abundance occurring after dawn and minimal levels at the end of the light period. The 5′-flanking region contains a functional G box (C/A-CACGTGGC) (Donald and Cashmore, 1990) which has demonstrated high-level constitutive expression in dicot and monocot plants (Ishige et al., 1999). These characteristics made the *Arabidopsis* rbcS promoter an interesting candidate for expression of tic107 in soybean. The 3′ end of the cassette is from the 3′ nontranslated region of the soybean alpha subunit of the beta-conglycinin gene (7S 3′) and provides the mRNA polyadenylation signals (Schuler et al., 1982) (Table 7).

To enable transport into the chloroplast, the *tic107* was fused to the targeting sequence from the *Arabidopsis* rbcS 1A (CTP1). The CTP1 was generated with the use of the chloroplast-targeting and mature NH2-terminal coding regions of the rbcS. The targeting sequence was modified by the addition of the first 24 amino acids of rbcS (MQVWPPIGKKKFETLSYLPDLTDS) because the efficiency of protein import is increased by addition of the sequence from the mature region of this protein (Stark et al., 1992). To facilitate removal of the remaining rbcS sequence from the tic107 protein, a second protease cleavage site (GGRVNCMQA) between these 24 amino acids of rbcS and the NH2-terminal Met of tic107 was added. We have generated nontargeted tic107 lines, and these lines on average accumulated much lower levels of tic107 (data not shown).

The *tic107* encodes a full-length Cry1A-like protein of 1178 amino acids, which when subjected to trypsinization yields an active N-terminal protein product or tryptic core of ≈600 amino acids in planta and in vitro (Fig. 4). The *cry1A* gene present in PV-GMBT01 was constructed by combining the first 1398 nucleotides of the *cry1Ab* gene (corresponding to amino acids 1–466) (Perlak et al., 1990) with nucleotides 1399 to 3524 of the *cry1Ac* gene (corresponding to amino acids 467–1178) (Fischhoff and Perlak, 1996). With the exception of 6 amino acid differences, the native Cry1Ab region is identical to the analogous region of the native Cry1Ac protein encoded by the *B. thuringiensis* subsp. *kurstaki cry1Ac* gene as described by Adang et al. (1985). The remaining portion of the *tic107* in PV-GMBT01 encodes an amino acid sequence that is identical to the Cry1Ac protein found in nature (Adang et al., 1985) with the exception of one amino acid at position 766 contained in the C-terminal region that is clipped away from the active portion of the protein during trypsinization. Thus, the encoded mature protein produced in these transgenic soybean lines are >99.4% identical to the naturally occurring Cry1Ac protein produced by *B. thuringiensis* subsp. *kurstaki*. However, this gene greatly differs from the wild-type gene found in the gram-positive prokaryote by increasing the G + C content via codons from highly expressed plant proteins, removing potential plant polyadenylation signal sequences (Dean et al., 1986), and removing ATTTA sequences that have

Table 6. Mortality (number and percentage) of fourth-instar velvetbean caterpillar larvae fed with transgenic *Bt* lines (862, 726, and 781) on different days after treatment (DAT) and mean foliage consumption per larva, compared with check (M-SOY 5826 is a commercial check). Bioassay II, Ponta Grossa, MONSOY Experimental Station.

	Mortality (No. larvae)						
Genotype	1 DAT	2 DAT	3 DAT	4 DAT	Total	Mortality	Mean foliage per larva
						%	cm^2
M-SOY5826	0	0	0	0	0	0	116.7
862	3	48	9	0	60	100	0.6
726	2	40	18	0	60	100	0.6
781	1	29	30	0	60	100	0.5

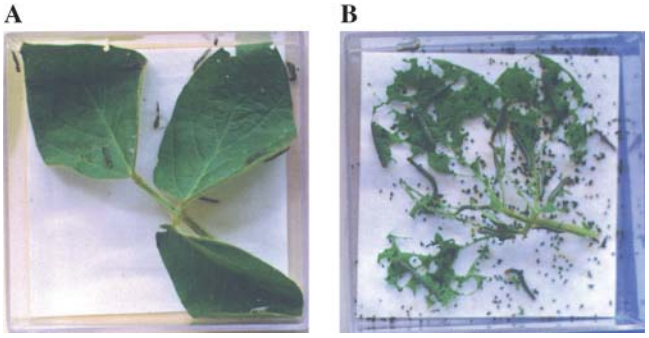

Fig. 5. Leaf feeding studies using velvetbean caterpillar. Defoliation of (A) transgenic *Bt* line 781 compared with (B) M-SOY5826 24 h after supplying foliage from these genotypes to third-instar velvetbean caterpillar larvae.

been shown to destabilize transcripts (Shaw and Kamen, 1986). Perlak et al. (1991) demonstrated that making these modifications to wild-type *Bt* genes could increase the levels of insect control proteins 100-fold. Although previous reports of transgenic soybean containing *Bt* genes have used a gene designed for high expression in plants, they were unable to generate plants with insecticidal proteins at the levels we are reporting (Stewart et al., 1996b; Adang et al., 1993).

Transgene Integration and Expression

Potential safety issue with multiple copy events or poorly characterized integration site of the transgene can be easily screened out because of the abundance of commercially viable lines allowing us to reduce the risk associated with unintended transgenes or sequences. Utilization of this plant expression cassette allowed identification of several efficacious events from the original transformation output of 87 events. We have shown this expression cassette (Fig. 1) to perform well independent of insertion sites. Apparently the tic107 expression cassette is not sensitive to position effects related to chromosomal locus and nearby DNA sequences. All transgenic soybean plants containing a functional transcriptional unit that expressed the *Bt* protein were found to be efficacious, which is one less multiplier in calculating efficiency. Many transgenic plants, as in the events described here, have a selectable marker included in the T-DNA. The inclusion of the selectable marker in the T-DNA aids in the selection of transgenic plants that are integrated in areas of the genome that are transcriptionally active, thus increasing the opportunity of generating efficacious plants (McCormac et al., 2001). Even under conditions in which the transgene was not associated or linked to a selectable marker we were able to efficiently and reproducible generate efficacious plants (MacRae et al., 2005). An optimized expression cassette in one organism, however closely related, does not necessarily suggest success in other organisms. Stewart et al. (1996b) generated their *Bt* soy plants using the same construct which produced transgenic canola with expression levels of up to 0.4%, and Singsit et al. (1997) have produced transgenic peanut with expression levels of 0.18%. We feel the underlying difference in the performance of an expression cassette has to be organism specific and related to differences in transcript regulation and the stability of transcripts and proteins. It can be hypothesized that transcription regulation is significantly affected by slight alterations in nucleotide sequence (primary structure) in the promoter, affecting binding of regulatory proteins that have evolved in greater affinity to native sequences. Provided binding occurs, the interaction of these regulators to the basal transcriptional unit (quaternary structure) may be suboptimal. In our view, the success of these complex relationships can only be empirically determined. As we become better at targeting expression to specific tissue through the plethora of genomics data available we will be able to limit the environmental exposure of these insecticidal proteins by expressing them primarily in tissue where feeding occurs. Historically, the first promoter used to express a *Bt* gene has been from a plant virus. These promoters have evolved to give high constitutive expression; however, they are not the most efficient means of expression. These advancements in our understanding of and use of tissue-specific promoters will minimize the superfluous expression of transgenes.

Table 7. Genetic elements present in T-DNA.

Element	Size	Function
	kb	
P-FMV	0.57	35S figwort mozaic virus promoter (Gowda et al., 1989).
AEPSPS/CTP2	0.31	DNA sequence for chloroplast transit peptide, isolated from *Arabidopsis thaliana* EPSPS; transit peptide directs the CP4-syn protein to the chloroplast, the site of aromatic amino acid synthesis (Shah et al., 1986).
CP4 syn	1.36	Synthetic version of the 5-enolpyruvulshikimate-3-phosphate synthase (EPSPS) sequence isolated from *Agrobacterium* sp. strain CP4 that imparts tolerance to glyphosate (Padgette et al., 1996).
E9 3′	0.65	The 3′ nontranslated region from rbcS E9 gene of *Pisum sativum* (pea) which terminates transcription and directs polyadenylation of the mRNA (Coruzzi et al., 1984).
P-ArabSSU1A	1.73	Promoter for *Arabidopsis thaliana* ribulose 1,5-bisphosphate carboxylase small subunit 1A gene (Krebbers et al., 1988).
CTP1	1.65	Chloroplast transit peptide from *Arabidopsis thaliana* ribulose 1,5-bisphosphate carboxylase small subunit 1A (TimKo et al., 1985).
tic107	3.53	The gene, which confers insect resistance from *Bacillus thuringiensis* subsp. k*urstaki*. The modified gene encodes an amino acid sequence that is 99.4% identical to the *cryIAc* gene as described by Fischhoff and Perlak (1996).
7S 3′	0.44	A 3′ nontranslated region of the soybean alpha subunit of the beta-conglycinin gene that provides the mRNA polyadenylation signals (Schuler et al., 1982).
P-NOS	0.3	Promoter from *Agrobacterium tumefaciens* pTiT37 for the gene encoding nopaline synthetase (Bevan et al., 1983)
nptII	1.0	The gene isolated from Tn5(Beck et al., 1982) which encodes for neomycin phosphotransferase type II. Expression of this gene in plant cells confers resistance to kanamycin and serves as a selectable marker for transformation (Fraley et al., 1983).
NOS 3′	0.27	A 3′ nontranslated region of the nopaline synthetase gene from *Agrobacterium tumefaciens*, which functions to terminate transcription and direct polyadenylation of the nptII mRNA (Depicker et al., 1982; Bevan et al., 1983)

Insect Bioassays

When generating transgenic *Bt* plants, the quality and quantity of transgene expression is of great importance. High-dose expression can reduce the risk of resistant genotype development. An alternative mode of action in combination with high dose may be an effective way to control the rare homozygous-resistant insect genotypes. High dose is only possible when expression cassettes are optimized for the crop species of interest. All three transgenic *Bt* lines exhibited a high degree of resistance against the three insects tested. No apparent differences in efficacy were observed between the three transgenic *Bt* lines against the three insect species. Looking at survival and tissue consumption by the different insects on the isogenic lines can assess the quality of the bioassay system. Velvetbean caterpillar, the most Cry1Ac-sensitive insect in the test, exhibited relatively higher mortality and lower tissue consumption in the controls than soybean looper or soybean podworm, both of which exhibited very low mortality and much higher tissue consumption. As a result, these latter two species are considered more suitable subjects for use in early assessments of insect resistance in transgenic *Bt* soybean. A significant result of this study is the high degree of efficacy observed against later instars of velvetbean caterpillar. Older larvae are generally more tolerant of both formulated *B. thuringiensis* insecticides (Roush, 1994; Ali and Young, 1996; Massé et al., 2001) and purified or transgenically-expressed endotoxins (Bai et al., 1993; Halcomb et al., 1996; Ashfaq et al., 2000; Henneberry and Jech, 2000; Parker et al., 2000) than neonates. The achievement of resistance against older, presumably more tolerant larvae is undoubtedly a result of the high tic107 expression levels achieved in these *Bt* soybean lines.

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