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Genetic Transformation, Recovery, and Characterization of Fertile Soybean Transgenic for a Synthetic *Bacillus thuringiensis cryIAc* Gene[1]

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Somatic embryos of Jack, a *Glycine max* (L.) Merrill cultivar, were transformed using microprojectile bombardment with a synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (*Bt cryIAc*) driven by the 35S promoter and linked to the *HPH* gene. Approximately 10 g of tissue was bombarded, and three transgenic lines were selected on hygromycin-containing media and converted into plants. The recovered lines contained the *HPH* gene, but the *Bt* gene was lost in one line. The plasmid was rearranged in the second line, and the third line had two copies, one of which was rearranged. The CryIAc protein accumulated up to 46 ng mg^{-1} extractable protein. In detached-leaf bioassays, plants with an intact copy of the *Bt* gene, and to a lesser extent those with the rearranged copy, were protected from damage from corn earworm (*Helicoverpa zea*), soybean looper (*Pseudoplusia includens*), tobacco budworm (*Heliothis virescens*), and velvetbean caterpillar (*Anticarsia gemmatalis*). Corn earworm produced less than 3% defoliation on transgenic plants, compared with 20% on the lepidopteran-resistant breeding line GatIR81-296, and more than 40% on susceptible cultivars. Unlike previous reports of soybean transformation using this technique, all plants were fertile. To our knowledge, this is the first report of a soybean transgenic for a highly expressed insecticidal gene.

Three of the four (*Glycine max* [L.] Merrill) economically significant insect pests of soybeans are lepidopterans (Adams et al., 1988) and are especially important in areas with long growing seasons, such as the southeastern United States (Hudson et al., 1993). These insect species, SBL, VBC, and CEW, are responsible for up to 89% of the economic insect damage in the southeastern United States, depending on the year (Hudson et al., 1993). As a result of increased insect-feeding pressure, fewer available effective insecticides, and public pressure for reduced pesticide use, transgenic plants are forecasted to play an important role in future integrated pest management in many crop plant species (Adang et al., 1988; Williams et al., 1992).

The bacterium *Bt* is common in the environment. It has been used as a biological control agent against lepidopteran insects for more than 50 years (reviewed by Tabashnik, 1994). The class of compounds responsible for insecticidal activity are crystalline proteins, also known as cry proteins or δ-endotoxins, whose mode of action is the disruption of midgut cellular membranes. In the midgut, endotoxins are proteolytically converted into polypeptides, which bind to glycoprotein receptors and disrupt osmotic processes (Adang, 1991).

One of the primary advantages of using *Bt* genes for insect control in transgenic plants is the specific insecticidal action toward certain insect orders (e.g. *Bt* CryI is specifically toxic to the order Lepidoptera), and therefore beneficial insects, birds, and mammals (including humans) are not harmed. *Bt* sprays have limited field efficacy, since they may be washed off of leaves by rain, and are most effective on young larvae. Therefore, spraying must be synchronized with imminent weather conditions and susceptible larval stages. These drawbacks of *Bt* sprays are not an issue with *Bt* transgenic plants. Because of these key advantages, it is expected that *Bt* will be engineered into nearly all major crop plants (Brattsten, 1991; McGaughey and Whalon, 1992). To date there have been 11 plant species transformed with *Bt* and field tested: *Amelanchier*, apple, canola, corn, cotton, *Populus*, potato, rice, tobacco, tomato, and walnut (APHIS permits, Feb., 1995). In addition, a search of the literature reveals that additional species have also been transformed with *Bt*: cabbage, white clover, common bean (Raybould and Gray, 1993), chrysanthemum (van Wordra-

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Abbreviations: ANOVA, analysis of variance; *Bt*, *Bacillus thuringiensis*; CEW, corn earworm, *Helicoverpa zea* Boddie; HPH, hygromycin phosphotransferase gene; FN, Finer and Nagasawa medium; MSD20, Murashige-Skoog salts, B5 vitamins, 3% Suc, 20 mg/L 2,4-D, pH 5.8; SBL, soybean looper, *Pseudoplusia includens* Walker; T_0, primary transgenic plants; T_1, progeny from T_0 transgenic plants; T_2, progeny from T_1 plants; TBW, tobacco budworm, *Heliothis virescens* Frabricius; VBC, velvetbean caterpillar, *Anticarsia gemmatalis* Hübner.

gen et al., 1993), cranberry (Serres et al., 1992), and soybean (Parrott et al., 1994). There is such great interest in *Bt*-protected transgenic plants that several researchers are already formulating strategies to prevent the rapid evolution of resistant insect biotypes (Tabashnik, 1989, 1994; Brattsten, 1991; Van Rie, 1991; McGaughey and Whalon, 1992; Alstad and Andow, 1995). Strategies include using rotations, refugia, mosaics, and multiple transgene combinations (pyramiding).

The majority of plant transformations using *Bt* have been performed with native genes (Barton et al., 1987; Vaeck et al., 1987; Perlak et al., 1990; Mehra-Palta et al., 1991; Murray et al., 1991; Hoffmann et al., 1992). Since plants do not have many of the tRNAs required by the bacterial gene codons, transgenic plants often express these native genes at low levels and do not kill insects that feed on them (Adang et al., 1993). For this reason, there has been much interest in the use of "codon-optimized" synthetic *Bt* genes for transgenic plant insect control (Perlak et al., 1990; Adang et al., 1993; Koziel et al., 1993). For example, the synthetic *Bt cryIII* transcript constituted more than 0.1% of the total mRNA in transgenic potato, compared with undetectable amounts using the native gene (Adang et al., 1993).

The objective of this study was to produce a soybean plant transgenic for synthetic *Bt cryIAc*. We describe the tissue culture and transformation procedures and the molecular and biotic characteristics of the *Bt* soybean that was produced.

MATERIALS AND METHODS

Gene Design and Plasmid Construction

The synthetic *Bt cryIAc* gene, patterned after the *Bt* ssp. *kurstaki cryIAc* sequence, was designed for high expression in plants (GenBank accession no. U63372) using methods published by Adang et al. (1993). The 1.8-kb truncated synthetic *Bt* gene was cloned into the plant expression vector pIC35/A (Murray et al., 1991). The 3.4-kb cassette, including the cauliflower mosaic virus 35S promoter, synthetic *Bt*, and the open reading frame 25 terminator, was subcloned into the *Bgl*II restriction site of the pSG3525 plasmid. This vector contains an HPH-selectable marker driven by the 35S promoter and orf25 terminator (Murray et al., 1991). The synthetic vector was renamed pSG/Bt (Fig. 1) (Singset et al., 1996). To monitor the efficiency of the shots, pGUSINT, a second plasmid with an intron-GUS construct (Vancanneyt et al., 1990), was also used in equimolar mixtures with pSG/Bt.

Tissue Culture, Transformation, and Regeneration

Somatic embryos of Jack, a *Glycine max* (L.) Merrill cultivar with high embryogenic capacity, were induced from immature cotyledons, proliferated, and maintained as described by Bailey et al. (1993a). Approximately 90 d after induction and 1 week after liquid suspensions were subcultured, clumps of globular-stage embryos were bombarded. The average clump diameter was 2 mm. *Escherichia coli* strain DH5α harboring the plasmids of interest was

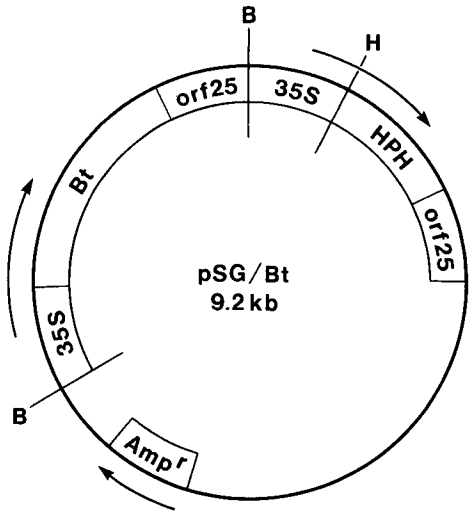

Figure 1. pSG/Bt, Synthetic *Bt cryIAc* construct used in particle bombardment. B, *Bgl*II, H, *Hin*dIII restriction sites. Arrow indicates direction of transcription. Amp^r, Ampicillin resistance is used as a bacterial marker.

grown separately in Luria broth (Sambrook et al., 1989), and plasmid DNA was isolated using an alkaline lysis/PEG purification procedure (Yeung and Lau, 1993). Plasmid DNA (pGUSINT; Vancanneyt et al., 1990) and pSG/Bt were suspended at equimolar amounts in a final concentration of 1 μg μL^{-1} and precipitated onto 1-μm gold particles (Gordon-Kamm et al., 1990). The Bio-Rad PDS 1000H was used for bombardment. Each plate was bombarded twice using 650-psi rupture discs supplied by Bio-Rad in a 700-mm mercury vacuum. First, embryos were transferred to an open Petri dish and desiccated for 15 min and then mashed gently with a spatula (Vain et al., 1993). Ten plates of somatic embryos (1 g per plate) were bombarded. Following bombardment, embryos continued to dry for 30 min in a covered Petri dish (Vain et al., 1993). The embryos were subsequently transferred to MSD20 medium (Bailey et al., 1993a). Next, the day after bombardment, the clumps from each plate were divided and transferred onto two selection plates containing MSD20 medium supplemented with 25 mg L^{-1} hygromycin. That same day, a GUS histochemical assay was performed on 50 somatic embryo clumps per shot plate (Jefferson, 1988). GUS staining is indicative of relative frequency of transformation based on transient expression levels (Jefferson, 1988). Transfers to fresh medium were made every 3 weeks.

At week 6, surviving embryogenic clusters were transferred to liquid FN medium (Finer and Nagasawa, 1988) without hygromycin. At this point putative transgenic cell lines were separated and tracked. Cells were allowed to acclimate for 4 weeks with a transfer to fresh medium after 2 weeks. Then, the clusters were transferred to FN containing 25 mg L^{-1} hygromycin for 2 weeks of final selection. The transgenic somatic embryos were germinated and converted using previously published methods (Bailey et al., 1993a). The T_0 plants were grown in 4-L pots in a sand:loam:bark mixture (1:1:1, w/w; Hyponex Maryville, OH)

under 23-h photoperiods (for vegetative growth) for 2 months in a glass greenhouse. Flowers were induced using 12-h photoperiods for the duration of the plants' life cycle. The T_1 plants were grown under similar conditions. We tested for Mendelian segregation by performing PCR analysis using *Bt* transgene-specific primers (bases 200–219 5′-ATTTGGGGAATCTTTGGTCC-3′; bases 789–770 5′-ACAGTACGGATTGGGTAGCG-3′) and performed a chi-squared test on T_2 plants (Zar, 1984).

DNA Blot Analysis

T_0 and T_1 plants were tested for stable integration of the *HPH* and *Bt* transgenes using DNA blot analysis. Plant genomic DNA was extracted using a miniprep (Stewart and Via, 1993) with modifications. Fresh leaf tissue (0.125 g) was placed in 500 μL of extraction buffer (2% [w/v] hexadecyltrimethyammonium bromide, 1.42 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0, 2% [w/v] PVP-40, 5 mM ascorbic acid, 4.0 mM diethyldithiocarbamic acid; Sigma) at room temperature in a microcentrifuge tube. Just prior to homogenization 6 μL of 2-mercaptoethanol was added to each 1 mL of buffer. Leaves were homogenized using a pellet pestle (BioVentures, Murphreesboro, TN) attached to a cordless drill. Immediately following homogenization (500 rpm for 45 s), the homogenate was extracted with 500 μL of chloroform:isoamyl alcohol (24:1, v/v). This mixture was gently inverted 10 times and microcentrifuged (5,000*g* at 22°C) for 5 min to separate phases. The upper, aqueous, DNA-containing phase was transferred to a fresh microcentrifuge tube, precipitated with 2.5 volumes of ethanol for 5 min at 4°C, and centrifuged (14,000*g* at 22°C) for 10 min. The pellet was washed once with 70% ethanol and centrifuged as above. The pellet was air-dried and resuspended in water. DNA was quantified using a minifluorometer (TKO 100, Hoefer Scientific Instruments, San Francisco, CA). Eight micrograms of plant genomic DNA and 20 pg of pSG/Bt were digested using *Bgl*II, which was used to cut out the 3.4-kb *Bt* insert (with promoter and terminator) to test for plasmid rearrangement in transgenic plants, or *Hin*dIII, which was used to show copy number. DNA was electrophoresed on 1% agarose gels, and DNA blot analysis was performed (Evans et al., 1994). Probes were ^{32}P-labeled using random primers (GIBCO-BRL). The *Bt cryIAc* 590-bp probe was produced by PCR using the primers described in the previous section. In addition, blots were probed with an *HPH*-coding region PCR fragment. DNA blots were probed and washed at 65°C. Autoradiograms were allowed to develop at −70°C between 1 and 5 d.

Protein Expression Analyses

Protein was extracted from leaves using a miniprep method (Adang et al., 1993). Fresh leaf tissue (0.2 g) was homogenized in 400 μL of 0.1 N NaOH using a Kontes (Vineland, NJ) pellet pestle and cordless drill in a microfuge tube. After the sample was incubated for 30 min on ice, 80 μL of 1 M Tris-HCl (pH 4.5) was added for neutralization. Each sample was clarified by centrifugation at 14,000*g* at 22°C. A preliminary ELISA was performed on T_0 plants using a double-sandwich antibody procedure (Adang et al., 1993). Protein from T_1 plants was analyzed using SDS PAGE, protein transfer, and immunoblot assays, which were performed according to published methods (Pratt et al., 1986). Fifty micrograms of protein was added to each well for analysis. The *Bt* CryIAc protein used for standard curves and for the production of specific polyclonal antibodies used in immunodetection was a wild-type protein isolated from *Bt* var *kurstaki* HD-73 as described by Garczynski et al. (1991). The wild-type protein had the same putative amino acid sequence as the synthetic gene product. We immunostained the blot with the following antibodies in sequence: rabbit anti-*Bt* serum (1:6,000), goat anti-rabbit (Sigma), and rabbit anti-goat/alkaline phosphatase (Sigma). The signal was detected by exposing the blot to nitroblue tetrazolium/bromochlorolindolyl phosphate substrate (Sigma) for approximately 3 min.

Insect Bioassays

Detached leaf insect bioassays were performed using previously described containers and methods (Parrott et al., 1994). Preliminary assays on T_0 plants were performed by placing 10 neonate larvae on a detached leaf from each of four clonal plants from each of three transgenic lines and one nontransgenic control line. The insect species tested individually or separately were TBW and CEW. The factorial treatment design included 2 insect species × 4 lines × 4 clonal replicates per line, in a completely randomized experiment ($n = 32$). The trial duration was 6 d. At the end of the trial, we estimated defoliation using a computer image analysis system (Hargrove and Crossley, 1988). Because of small sample sizes, the data were analyzed with a Kruskal-Wallis test, a nonparametric ANOVA analog, and the corresponding multiple comparison algorithm (Hollander and Wolfe, 1973), with which data were arranged according to insect species and analyzed.

To achieve better estimates of line effects and to compare transgenic plant antibiosis with known resistant and susceptible soybean lines, T_1 plants were bioassayed using methods described above. Using DNA blot band intensity confirmed by PCR analysis on T_2 plants, we estimated whether each T_1 plant was homozygous positive, hemizygous, or homozygous null for the transgene. We then divided plants of each of two *Bt* transgenic lines (denoted 7b and 10c) into one of the three classes mentioned above (i.e. 7bBT/BT, 7bBT/0, and 7b0/0). We used only *HPH*-positive plants in the *HPH* transgenic line (8a). For a control, we used cv Jack plants that had been propagated via the same tissue culture regeneration scheme as the transgenic plants. The lepidopteran-susceptible cultivar used was Cobb, and the resistant breeding line was GatIR81-296 (Beach and Todd, 1987; Parrott et al., 1994). One leaf from each of six plants of each class was exposed to CEW. The experiment was sequentially replicated three times. Five response variables were taken. First, the percentage of defoliation of leaves and the insect survivorship were tabulated. Then, physiological inhibition was determined by collecting surviving insects and measuring head capsule width, body

length, and weight. The experiment included 10 plant classes (susceptible and resistant cultivars, lines [transgene status]) × 6 plants per class × 3 replicates per plant in time (n = 180). A two-way ANOVA was performed using plant classes and plant identity (replication) as the classification variables. Multiple comparisons were made using Tukey's honestly significant difference (SAS Institute, 1990).

To better estimate transgenic plant antibiosis against other economically important soybean-defoliating lepidopterans, we performed a third assay using identical methods. Detached leaves of six plants each of 7bBT/BT and 7b0 were bioassayed as above, using two insect species (singularly), with one replicate per plant in time. The insects tested were SBL and VBC. Thus, the experiment included 2 plant lines × 6 plants per line (n = 12). The data were arranged according to insect species and analyzed using a Kruskal-Wallis test, a nonparametric one-way ANOVA analog (Hollander and Wolfe, 1973).

RESULTS

Plant Transformation

In the GUS assay, 27% of the embryos had one or more blue spots with a mean of 1.84 blue spots per embryo clump. These data include only those plates that showed any GUS staining. None of the clumps from the remaining six plates had any blue foci. At the end of the initial selection period (6 weeks) 50 lines of putative transformants were identified by their bright green and embryogenic appearance. After these lines were proliferated on FN 42 lines remained alive and embryogenic. At the end of the final selection regime (FN plus 25 mg L^{-1} hygromycin) only four lines remained green and embryogenic. After these clones were further proliferated, only three (7b, 8a, and 10c) of the four could be converted into plants. The nomenclature we used to label the lines reflects the plate/shot number (e.g. 7) and clonal identity within a shot (e.g. b). The only lines recovered from selection and transgenic plants came from plates identified to have members that had blue GUS spots. The remaining line (8h) was found to be *Bt*-positive and also transgenic for GUS based on histochemical assay and DNA blot analysis (data not shown). Plants from two (8a and 10c) of the three lines developed normally and had full seed set. We arbitrarily recovered 20 plants of line 7b, 25 plants of line 8a, and 50 plants of line 10c primary transgenic plants. Whereas 90% of the 8a and 10c plants survived, 19 of the 20 7b plants died following the appearance of an epiphytotic of *Rhizoctonia* in the greenhouse. Concurrently, all of the 7b somatic embryos used for cell line maintenance died and the remaining transgenic cell cultures continued to proliferate.

Transgenic plants, including the single 7b plant, were allowed to self-pollinate, and were all fully fertile and produced ample seed. We randomly chose one clonal replicate of the original lines as a representative of that line (except for the 7b singleton) and germinated 30 seeds of each. In the cases of the 8a and 10c progeny, transgenes segregated in Mendelian (3:1) fashion (χ^2 at $\alpha > 0.05$), but the 7b line had an obvious excess of homozygous nulls ($P < 0.05$), in which there were 18 transgene positive and 11 transgene negative plants. However, the T_1 plants that survived were morphologically normal and fertile. There were no significant differences in seed yield among 7b and 10c T_1 lines with regard to transgene status (ANOVA, $P > 0.05$).

DNA Analysis

DNA blot analysis on T_0 plants showed that line 7b contained two copies each of the transgenes (*Bt* [Fig. 2] and *HPH* [data not shown]), one rearranged (evident from a missing restriction site) and one intact copy. Line 10c had one rearranged copy of the transgenes, and line 8a contained the *HPH* but not the *Bt* gene. Furthermore, *Hin*dIII digestion confirmed that all transformation events were independent (Fig. 2). DNA blot analysis on T_1 plants revealed that all transgenes were stably inherited (Fig. 3). Furthermore, the two copies of transgenes in line 7b were linked (Fig. 3). PCR analysis verified the transgenic status and that the 7b line indeed had an excess of homozygous nulls (Fig. 4).

Protein Expression

Bt expression levels for the T_1 plants ranged from 0 (8a) to 46 ng mg^{-1} (7bBT/BT) (Table I). The 7b line demonstrated a dose effect for Cry associated with zygosity, in which the homozygotes had approximately twice the expression as heterozygotes (Table I). All 10c plants had lower expression than the 7b plants (Table I). Instead of the expected 60-kD protein that was present in the 7b and Cry standards, a truncated 48-kD peptide was present in the 10c transgenic plants (Fig. 5). However, in addition to the 60-kD band, there were 44- and 35-kD bands present in approximately the same proportions as the 60-kD band in 7b. These bands were absent in the control lanes.

Insect Bioassays

The initial insect bioassays using T_0 plants showed that the *Bt*-susceptible TBW was totally controlled by plants

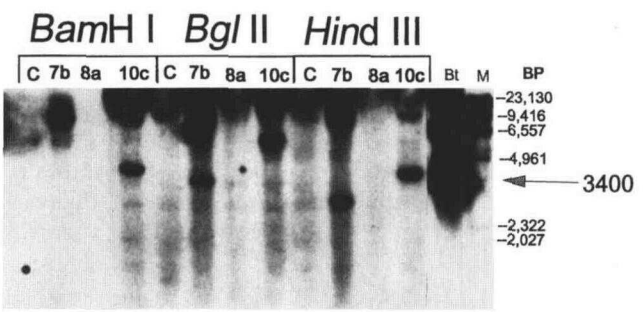

Figure 2. DNA blots of the T_0 plants probed with the *Bt cryIAc* PCR product. *Bgl*II cuts the *Bt*-containing insert to yield a 3400-bp fragment in pSG/Bt. *Hin*dIII has one unique site in pSG/Bt. These results show two integrated copies of the *Bt cryIAc* transgene in line 7b (one rearranged and one intact copy) and one in line 10c (a rearranged copy). The probe did not hybridize to the transgenic 8a line, demonstrating that the *Bt* gene was not integrated. Lane M, Molecular weight marker.

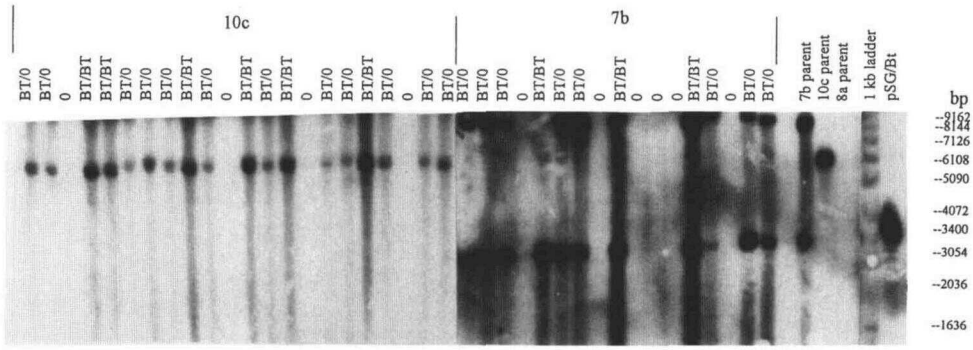

Figure 3. DNA blots of T_1 *Bt cryIAc*-containing transgenic plants probed with the *Bt CRYIA(c)* PCR product, in which DNA was digested with *Bgl*II. Mendelian segregation (3:1) is evident in the 10c line but not the 7b line, which has an excess of homozygous nulls. Note that the rearranged copy and the intact copy of the transgene are linked in the 7b line. Densitometric estimates validated by PCR analysis of T_2 progeny plants (see Fig. 4) yielded the lane designations.

with the *Bt* gene (no defoliation versus 29% defoliation in control plants), whereas defoliation from the relatively *Bt*-resistant CEW was significantly affected ($P = 0.05$) by the concentration of CRY toxin in the plants (7b, 8%; 10c, 19%; nontransgenic control, 46% defoliation).

The results of the insect bioassays using T_1 plants corresponded with those of the T_0 assays. Bioassays of CEW demonstrated the comparative performance of the various *Bt*-transgenic soybean lines relative to known resistant and susceptible lines. Larval head capsule width and body length were invariant among lines (Table I). Larval weights were significantly different between 7b plants and the remaining lines tested (Table I). For insect survivorship, only the 7bBT/BT and 7bBT/0 lines showed significant differences ($P < 0.05$) from the others, in which less than 1 of 10 insects survived, compared with 4.4 to 6.2 surviving insects on other soybean lines (Table I). The lepidopteran-resistant breeding line GatIR81-296 had intermediate antibiosis activity (4.1 insects surviving). Although statistically not significantly different from the *Bt*-transgenic lines, it was biologically more similar to the non-7b transgenic lines. The defoliation data revealed similar trends, in which 7b transgenic leaves remained nearly intact, whereas susceptible plants suffered 26.4 to 39.6% defoliation (Table I). Intermediate defoliation occurred on the 10cBT/BT and 10cBT/0 transgenic lines as well as the GatIR81-296 line (Fig. 6).

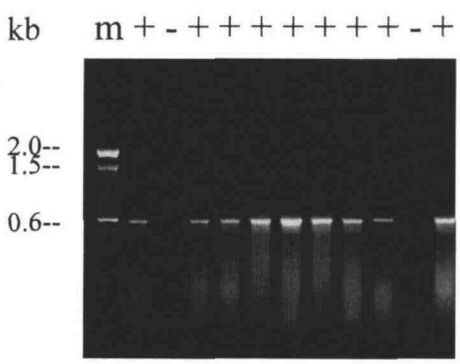

Figure 4. PCR analysis of T_2 seed using *Bt cryIAc* primers from bases 200 to 219 5′-ATTTGGGGAATCTTTGGTCC-3′ and bases 789 to 770 5′-ACAGTACGGATTGGGTAGCG-3′, to yield the expected 560-bp fragment. T_2 seed that are PCR-null indicate that the parent T_1 plant was hemizygous for the transgene. m, Molecular weight markers; +, transgenic; −, nontransgenic.

We used smaller sample sizes to directly compare T_1 isogenic transgenic and non-transgenic 7bBT/BT and 7b0/0 for two other soybean lepidopteran pests, SBL and VBC. As with the assays of CEW, the insect morphology data were generally less statistically significant than that for insect survivorship and leaf defoliation (Table II). The 7bBT/BT plants were completely protected from the *Bt*-susceptible VBC, in which all larvae were killed (Table II). In comparison, the transgenic plants were almost but not totally protected against the *Bt*-resistant SBL. However, the results from the SBL trial were similar to those of the CEW trial and showed statistically significant differences.

DISCUSSION

Plant Transformation and Recovery

Genetic transformation of soybean remains far from routine. However, one method in particular has produced several stably transformed transgenic soybean plants (McCabe et al., 1988; Christou 1990; McCabe and Christou, 1993). With this procedure, embryonic axes are bombarded by an electric discharge apparatus followed by GUS histochemical staining and visual selection of transgenic shoots. This procedure is contingent on the use of an electric discharge particle acceleration device, which is not generally available. Other laboratories have explored less costly means to transform soybean, including *Agrobacterium*-mediated transformation of cotyledonary nodes (Townsend and Thomas, 1993) and the method we used, namely microprojectile bombardment of somatic embryos (Finer and McMullen, 1991; Sato et al., 1993; Parrott et al., 1994). The latter procedure has the draw backs of reduced fertility or sterility of transgenic lines. The use of this procedure to obtain fully fertile plants represents an important advance in soybean transformation technology.

In addition, there is large variability in recovery rate among untransformed genotypes (Bailey et al., 1993b) and also among transgenic lines of the same genotype (this

Table I. *Summary of data of transgenic soybean and antibiosis trials using CEW*

Ten neonate larvae were applied to a detached leaf for 5 d. Insects were tallied and measured, and leaf defoliation was estimated. This experiment was repeated three times. Different letters in columns indicate significant differences at $\alpha = 0.05$ using Tukey's honestly significant difference. $n = 18$, except 10cBt/Bt and 7bBt/Bt, where $n = 15$.

Line	Description	Transgene Status	Bt Protein	Live Larvae	Head Capsule Width	Body Length	Larval Wt	Defoliation
			ng/mg	*No.*	*mm*	*mm*	*mg*	*%*
Jack	Cell culture	None	0	5.5 a	5.9 a	40.3 a	47.3 a	39.6 a
8a	HPH	Variable	0	6.2 a	5.9 a	41.1 a	57.5 a	30.2 a
10c0	Rearranged *Bt*	Null	0	5.5 a	6.1 a	40.1 a	51.7 a	27.4 a
10cBt/0	Rearranged *Bt*	Hemizygous	1.7	4.4 a	5.7 a	38.0 a	45.8 a	21.4 ab
10cBt/Bt	Rearranged *Bt*	Homozygous	13.2	4.6 a	5.3 a	34.2 a	35.8 a	23.3 ab
7b0	Intact *Bt*	Null	0	4.9 a	5.4 a	36.7 a	36.2 a	28.6 a
7bBt/0	Intact *Bt*	Hemizygous	23.5	0.7 b	3.6 a	23.7 a	12.0 b	2.4 b
7bBt/Bt	Intact *Bt*	Homozygous	45.8	0.4 b	2.9 a	21.7 a	10.1 b	1.1 b
GatIR81-296	Insect resistant	None	0	4.1 ab	5.2 a	35.3 a	32.9 a	19.8 ab
Cobb	Insect susceptible	None	0	4.7 a	5.4 a	39.8 a	70.9 a	26.4 a

manuscript). However, we observed that among the four lines presented in this study those lines that regenerated most easily (namely 8a and 10c) corresponded to those that were most morphologically normal. In contrast to earlier studies in which primary transformants had decreased growth rates, abnormal leaf morphology, or decreased fertility (Finer and McMullen, 1991; Parrott et al., 1994), and progeny, if any were obtained, that lost fertility in successive generations, the transgenic lines produced in this study were morphologically normal and did not lose fertility. For example, four generations of 10c and three generations of 7b lines have been produced in the greenhouse to date with no morphological defects evident and no decrease in fertility. However, the T_0 7b plants suffered disproportionate mortality (19 of 20 plants died from *Rhizoctonia* infection in the greenhouse), 7b T_1 transgenic seeds did not germinate on growth regulator-free Murashige-Skoog medium containing hygromycin, and 7b had skewed segregation data with excessive homozygous nulls (see "Results").

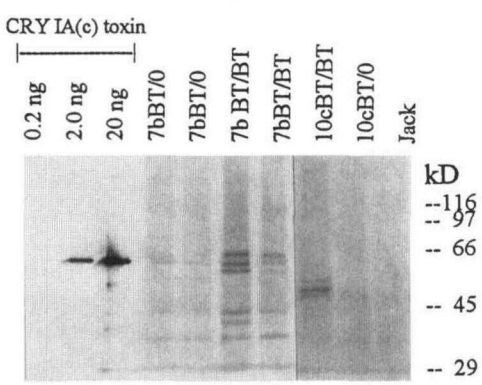

Figure 5. Protein blot of *Bt cryIAc*-transgenic plants showing typical amounts of immunostaining from homozygotes and hemizygotes for *Bt cryIAc*. Three specific bands close to the expected molecular mass and one to three bands of lower molecular masses immunostained in the 7b line. In the 10c line, which contains a rearranged version of the transgene, one lower-than-expected band immunostained. When we estimated expression levels in 7b, only the three high-molecular-mass bands were considered.

The extreme susceptibility to stress present in the original 7b plants has since segregated out, suggesting that this reduced viability may have been due to a mutation that occurred during the tissue culture process.

There were two methodological differences between the procedure presented here and earlier reports: cell age and selection regime (Finer and McMullen, 1991; Parrott et al., 1994). Young cell lines (approximately 3–4 months old) were bombarded, in contrast to cell lines that were 1 year old and older, as reported earlier. We used initial postbombardment selection of transgenic lines on solidified MSD20 medium instead of FN liquid, and selection was begun the day after instead of 1 to 2 weeks after bombardment. A priori, a potential advantage of this strategy compared with the liquid selection strategy is to promote slower growth of transgenic cells and slower death of nontransgenic cells. Since embryo clumps are statically placed on solidified medium, there is less opportunity for potentially toxic leachates of dying clumps to interfere with the growth of living clumps. However, it is unknown at this time whether this difference in the initial selection regime, the age of the cells bombarded, both, or neither accounts

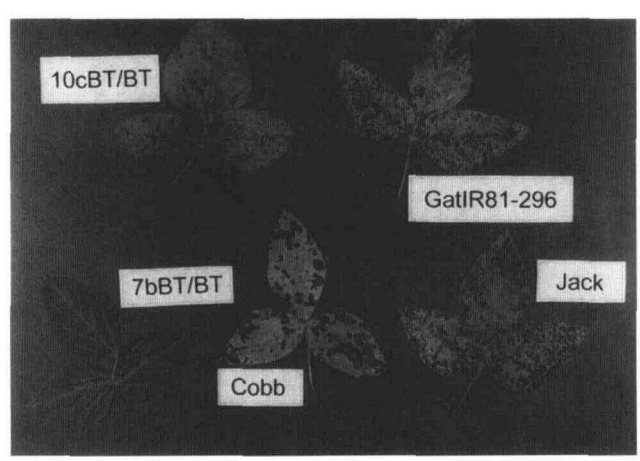

Figure 6. *Defoliation of soybean leaves by CEW. Detached leaves* were exposed to 10 CEW for 6 d. See Table I for line designations.

Table II. *Antibiosis trials using SBL and VBC with the most strongly expressing transgenics versus a segregant without the transgenes*

These tests were performed once, necessitating a nonparametric statistical Krustal-Wallis test. Comparisons were made in columns by insect species. See Table I for line descriptions.

Test Insect	Line	Live Larvae	Head Capsule Width	Body Length	Larval Wt	Defoliation
		No.	*mm*	*mm*	*mg*	*%*
SBL	7b0	4.0 P = 0.007	5.1 P = 0.055	45.3 P = 0.09	46.6 P = 0.26	15.5 P = 0.02
	7Bt/Bt	1.2	3.3	30.6	32.0	4.0
VBC	7b0	4.8 P = 0.002	5.6 P = 0.03	43.5 P = 0.07	31.5 P = 0.07	42.5 P = 0.002
	7Bt/Bt	0.0	0.9	8.2	8.3	0.0

for the morphological normality and fertility of the recovered plants.

Transgene Integration and Expression

In all of the transgenic cell lines the transgene integrated in a stable fashion and was inherited in progeny. However, at one point in the transformation process the plasmid containing the transgenes underwent a rearrangement. All three lines had some rearrangement occur, and only the 7b line retained an intact copy of the *Bt* transgene. In this line, the intact transgene was linked with the rearranged copy.

Protein blots (Fig. 5) showed that the 7b line contained bands of the expected 65-kD size, along with bands of slightly higher and lower molecular mass, and specific immunostained bands of between 35 and 45 kD. To determine whether the low-molecular-mass bands were the result of degradation during sample preparation, we spiked known amounts of bacterium-derived CryIAc toxin into nontransgenic homogenized leaf tissue. Since the spiked sample contained only the expected band, we may safely assume that the nontargeted endotoxin was modified in planta. The 10c line, which contained a rearranged version of the *Bt* transgene, contained an approximately 50-kD band that was unique to that line. Thus, the rearranged gene seems to be translated to a shortened peptide, and in turn, there is also less of this peptide than in the 7b line. However, the 7b line contains a rearranged transgene and an intact transgene. Thus, although it is not possible to now definitively attribute all observed immunostained peptide fragments to specific transgenes in 7b, we may safely assume that at least the expected-size *Bt* endotoxin protein observed in 7b was a product of the intact *Bt* transgene. Fujimoto et al. (1993) observed multiple CryIAb-specific bands of smaller molecular weight in transgenic rice. They attributed these truncated peptides to shortened mRNA species, possibly the result of cryptic polyadenylation regions in the transgene (Perlak et al., 1991; Fujimoto et al., 1993).

The levels of the synthetic *Bt* transgene product were very low in comparison with those in other reports of transgenic plants containing synthetic *Bt* transgenes. In our earlier transgenic soybean study (Parrott et al., 1994), expression levels were about 1 ng native CryIAb per milligram of total extractable protein. Although the expression levels in the current study are up to 40 times higher than in our previous work, the levels are 5 times less than those reported by Adang et al. (1993) for synthetic CryIIIA in transgenic potato and 40 times less than synthetic CryIA(b) in maize (Koziel et al., 1993). The reasons for the decreased expression are not clear. Using the same construct we (Stewart et al., 1996) have produced transgenic canola with expression levels of up 0.4%, and Singset et al. (1996) have produced transgenic peanut with expression levels of 0.18%. It is generally accepted that, when producing several independently transformed plants, expression levels are greatly variable. It is probable that the observed low expression level is simply the result of chance and low transformation efficiency and is not intrinsic to soybean itself. Therefore, as soybean transformation efficiencies continue to improve, plants with higher expressing *Bt* may be produced.

Insect Antibiosis

The soybean cv Jack was susceptible to all of the insects tested. Although not significantly higher, the percentage of defoliation was similar to that in Cobb, a well-documented susceptible cultivar. In several studies the insect-resistant breeding line GatIR81-296 was subject to between 0.52 and 0.88 as much defoliation compared with Cobb from CEW (Rowan et al., 1991, 1993). These studies have been performed in greenhouse, field enclosure, and field situations. Thus, the level of defoliation that occurred in our detached-leaf assay (GatIR81-296 had 0.75 defoliation compared with Cobb) approximates the median of the other comparisons. In addition, according to Parrott et al. (1994), who compared various antibiosis made with Cobb using VBC, soybean transformed with a native *Bt cryIAb* gene had 44% of the defoliation of Cobb, which was not significantly different than the GatIR81-296 resistance levels. In contrast, whereas the rearranged *Bt* transgenic line (10c) had similar resistance to CEW as GatIR81-296, the intact *Bt* soybean line (7b) had only 0.07 the defoliation as Cobb. Thus, the 7b line is an important improvement over natural resistance to insects in soybean.

To our knowledge this is the first report of soybean transformed with a biologically effective insect resistance gene. However, as is, these transgenic lines are not deployable in the southeastern United States, where lepidopteran damage warrants the deployment of *Bt* transgenic plants, since the survival levels of caterpillars are still high enough to foster the development of resistant insect populations. This problem could be especially acute, since *Bt* maize and cotton will also be grown in the area. Even if the plants produced *Bt* levels high enough to kill all target insects,

any prudent scenario for the deployment of *Bt* soybean should incorporate additional strategies to limit the selection of *Bt*-resistant insects. One strategy could consist of pyramiding *Bt* with proteinase inhibitor transgenes and/or introgressing the *Bt* transgene into the GatIR81-296 background, because the latter has an estimated four genes for lepidopteran resistance already (Rufener et al., 1989). Thus, the pyramiding strategy combined with insect-friendly refugia in space and time may delay the time for insects to acquire resistance to *Bt*. Utilization of *Bt* soybean in this framework may provide growers with an economic and environmentally desirable alternative to frequent insecticide applications used in standard soybean cultivation.

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LITERATURE CITED

Adams DB, McPherson RM, Jones DC, Horton DL, Todd JW (1988) Soybean insects. *In* GK Douce, RM McPherson, eds, Summary of Losses from Insect Damage and Costs of Control in Georgia, 1987, special publication 54. The Georgia Agricultural Experiment Station, Athens, GA, p 26

Adang MJ (1991) *Bacillus thuringiensis* insecticidal crystal proteins: gene structure, action, and utilization. *In* E Maramorosch, ed, Biotechnology for Biological Control of Pests and Vectors. CRC, Boca Raton, FL, pp 3–24

Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE (1993) The construction and expression of a *Bacillus thuringiensis cryIIIA* gene in protoplasts and potato plants. Plant Mol Biol **21:** 1131–1145

Adang MJ, DeBoer D, Endres J, Firoozabady E, Klein J, Merlo A, Merlo D, Murray E (1988) Manipulation of *Bacillus thuringiensis* genes for pest control. *In* DW Roberts, RR Granandos, eds, Biotechnology, Biological Pesticides, and Novel Plant-Pest Resistance for Insect Pest Management. Cornell University, Ithaca, NY, pp 31–37

Alstad DN, Andow DA (1995) Managing the evolution of insect resistance to transgenic plants. Science **268:** 1894–1896

Bailey MA, Boerma HR, Parrott WA (1993a) Genotype effects on proliferative embryogenesis and plant regeneration of soybean. In Vitro Cell Dev Biol **29P:** 102–108

Bailey MA, Boerma HR, Parrott WA (1993b) Genotype-specific optimization of plant regeneration from somatic embryos of soybean. Plant Sci **93:** 117–120

Barton KA, Whitely HR, Yang N-S (1987) *Bacillus thuringiensis* delta-endotoxin expressed in transgenic *Nicotiana tabacum* provides resistance to lepidopteran insects. Plant Physiol **85:** 1103–1109

Beach RM, Todd JW (1987) Resistance of the soybean breeding line GatIR 81-296 to foliar feeding by three *Spodoptera* sp. J Agric Entomol **4:** 193–199

Brattsten LB (1991) Bioengineering of crop plants and resistant biotype evolution in insects: counteracting coevolution. Arch Insect Biochem Physiol **17:** 353–267

Christou P (1990) Soybean transformation by electric discharge particle acceleration. Physiol Plant **79:** 210–212

Evans MR, Bertera AL, Harris DW (1994) The Southern blot: an update. Mol Biotechnol **1:** 1–12

Finer JJ, McMullen MD (1991) Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. In Vitro Cell Dev Biol **27P:** 175–182

Finer JJ, Nagasawa A (1988) Development of an embryogenic suspension culture of soybean (*Glycine max* Merrill.). Plant Cell Tissue Org Cult **15:** 125–136

Fujimoto H, Itoh K, Yamamoto M, Kyozuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Biotechnology **11:** 1151–1155

Garczynski SF, Crim JW, Adang MJ (1991) Identification of putative insect brush border membrane-binding molecules specific to *Bacillus thuringiensis* δ-endotoxin by protein blot analysis. Appl Environ Microbiol **57:** 2816–2820

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR Jr, Willetts NG, Rice TB, Mackey CJ (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell **2:** 603–618

Hargrove WW, Crossley DA Jr (1988) Video digitizer for the rapid measure of leaf area lost to herbivorous insects. Ann Entomol Soc Am **81:** 593–598

Hoffmann MP, Zalom FG, Wilson LT, Smilanick LD, Malyj LD, Kiser J, Hilder VA, Barnes WM (1992) Field evaluation of transgenic tobacco containing genes encoding *Bacillus thuringiensis* δ-endotoxin or cowpea trypsin inhibitor: efficacy against *Helicoverpa zea* (Lepidoptera: Noctuidae). J Econ Entomol **85:** 2517–2522

Hollander M, Wolfe DA (1973) Nonparametric Statistical Methods. John Wiley & Sons, New York

Hudson RD, Jones DC, McPherson RM (1993) Soybean Insects. *In* RM McPherson, GK Douce, eds, Summary of Losses from Insect Damage and Cost of Control in Georgia, 1992, special publication no 83. The Georgia Agricultural Experiment Station, Athens, GA

Jefferson RA (1988) Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol Biol Rep **5:** 387–405

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotechnology **11:** 194–200

McCabe D, Christou P (1993) Direct DNA transfer using electric discharge particle acceleration (ACCELL™ technology). Plant Cell Tissue Org Cult **33:** 227–236

McCabe DE, Swain WF, Martinell BJ, Christou P (1988) Stable transformation of soybean (*Glycine max*) by particle acceleration. Biotechnology **6:** 923–926

McGaughey WH, Whalon ME (1992) Managing insect resistance to *Bacillus thuringiensis* toxins. Science **258:** 1451–1455

Mehra-Palta A, Graves A, Kraemer M, Hill K, Eagan N (1991) Genetic transformation of *Brassica napus* and *Brassica rapa*. *In* Anonymous Rapeseed in a Changing World: GCIRC Congress, Saskatoon, Saskatchewan, Canada, pp 1108–1115

Murray EE, Rochleau TR, Eberle M, Stock C, Sekar V, Adang MJ (1991) Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol Biol **16:** 1035–1060

Parrott WA, All JN, Adang MJ, Bailey MA, Boerma HR, Stewart CN Jr (1994) Recovery and evaluation of soybean (*Glycine max* Merr.) plants transgenic for a *Bacillus thuringiensis* var. *kurstaki* insecticidal gene. In Vitro Cell Dev Biol **30P:** 144–149

Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT, Fischoff DA (1990) Insect resistant cotton plants. Biotechnology **8:** 939–943

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequence enhances plant expression of insect control protein genes. Proc Natl Acad Sci USA **88:** 3324–3328

Pratt LH, McCurdy DW, Shimazaki Y, Cordonnier M-M (1986) Immunodetection of phytochrome: immunocytochemistry, immunoblotting, and immunoquantitation. *In* HF Linskens, JF Jackson, eds, Modern Methods of Plant Analysis, Vol 4. Springer, New York, pp 50–74

Raybould AF, Gray AJ (1993) Genetically modified crops and hybridization with wild relatives: a UK perspective. J Appl Ecol **30:** 199–219

Rowan GB, Boerma HR, All JN, Todd J (1991) Soybean cultivar resistance to defoliating insects. Crop Sci **31:** 678–682

Rowan GB, Boerma HR, All JN, Todd JW (1993) Soybean maturity group effect on expression of resistance to lepidopterous insects. Crop Sci **33:** 433–436

Rufener GK II, St Martin SK, Cooper RL, Hammond RB (1989) Genetics of antibiosis resistance to Mexican been beetle in soybean. Crop Sci **29:** 618–622

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

SAS Institute (1990) SAS/STAT User's Guide, Version 6. SAS Institute, Cary, NC

Sato S, Newell C, Kolacz K, Tredo L, Finer J, Hinchee M (1993) Stable transformation via particle bombardment in two different sobyean transformation systems. Plant Cell Rep **12:** 408–413

Serres R, Stang E, McCabe D, Russell D, Mahr D, McCown B (1992) Gene transfer using electric discharge particle bombardment and recovery of transformed cranberry plants. J Am Soc Hortic Sci **117:** 174–180

Singset C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P (1996) Expression of *Bacillus thuringiensis cryIA(c)* gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Transgenic Res (in press)

Stewart CN Jr, Adang MJ, All JN, Raymer PL, Ramachandran S, Parrott WA (1996) Insect control and dosage effects in transgenic canola containing a synthetic *Bacillus thuringiensis CryIAc* gene. Plant Physiol **112:** 115–120

Stewart CN Jr, Via LE (1993) A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques **14:** 748–751

Tabashnik BE (1989) Managing resistance with multiple pesticide tactics: theory, evidence, and recommendations. J Econ Entomol **82:** 1263–1269

Tabashnik BE (1994) Evolution of resistance to *Bacillus thuringiensis*. Annu Rev Entomol **39:** 47–79

Townsend JA, Thomas LA (1993) An improved method of *Agrobacterium*-mediated transformation of cultured soybean cells, patent WO 94/02620. World Intellectual Property Organization

Vaeck M, Reynaerts A, Höfte H, Jansens S, De Beuckeler M, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. Nature **238:** 33–37

Vain P, Keen N, Murillo J, Rathus C, Nemes C, Finer JJ (1993) Development of the particle inflow gun. Plant Cell Tissue Org Cult **33:** 237–246

Vancanneyt G, Schmidt R, O'Connor-Sanchez A, Willmitzer L, Rocha-Sosa M (1990) Construction of an intron-containing marker gene: Splicing on the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation. Mol Gen Genet **220:** 245–250

Van Rie J (1991) Insect control with transgenic plants: resistance proof? Trends Biotechnol **9:** 177–179

van Wordragen MF, Honee G, Dons HJM (1993) Insect resistance chrysanthemum calluses by introduction of a *Bacillus thuringiensis* crystal protein gene. Transgenic Res **2:** 170–180

Williams S, Friedrich L, Dincher S, Carozzi N, Kessmann H, Ward E, Ryals J (1992) Chemical regulation of *Bacillus thuringiensis* δ-endotoxin expression in transgenic plants. Bio/Technol **10:** 540–543

Yeung MC, Lau AS (1993) Fast and economical large-scale preparation of high-quality plasmid DNA. Biotechniques **17:** 381–382

Zar JH (1984) Biostatistical Analysis. Prentice-Hall, Englewood Cliffs, NJ