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RECOVERY AND EVALUATION OF SOYBEAN PLANTS TRANSGENIC FOR A *BACILLUS THURINGIENSIS* VAR. *KURSTAKI* INSECTICIDAL GENE

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SUMMARY

Lepidopteran insects are major defoliating pests of soybean in the southeastern United States. Soybean plants transgenic for a native *cryIA(b)* gene from *Bacillus thuringiensis* var. *kurstaki* HD-1 were obtained. Embryogenic cultures were induced by plating cotyledons on a Murashige and Skoog-based medium supplemented with 40 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D). The embryogenic cultures were maintained in liquid medium containing 5 mg/liter 2,4-D. These cultures were subjected to microprojectile bombardment, followed by selection on 50 mg/liter hygromycin. Resistant embryogenic cell lines were transferred to growth regulator-free medium to permit recovery of mature somatic embryos. After a desiccation period, the somatic embryos were returned to growth regulator-free medium for conversion into plants. Southern hybridization analysis verified transformation. Feeding assays of T_1 plants from one cell line deterred feeding, development, and survival of velvetbean caterpillar at a level comparable to that of GatIR81-296, a soybean breeding line with a high level of insect resistance. Reduced feeding on T_1 plants correlated with the presence of the transgene.

Key words: Bacillus thuringiensis; insect resistance; microprojectile bombardment; somatic embryogenesis; soybean; transformation.

INTRODUCTION

Soybean (*Glycine max* [L.] Merr.) growers in the Southern United States annually suffer economic losses to defoliating insects. Three of the major pests are soybean looper, corn earworm, and velvetbean caterpillar, which together account for 89% of the total economic damage from insects on the soybean crop in the southeastern United States (31). Pesticides have been used traditionally to control these insects. However, soybean looper and corn earworm control is threatened as these species evolve resistance to pyrethroids (48). With a need to increase grower profits and reduce the risk of pesticide residues in the food chain, biologically based control measures are being explored.

The bacterium *Bacillus thuringiensis* (*Bt*) has been used as a biological insecticide for over 30 yr (22). The active component of *Bt* consists of subunits of insecticidal crystal proteins (cry proteins) aggregated into parasporal crystals. When susceptible insect larvae ingest *Bt* crystals they quickly stop feeding and die in several days due to the cytotoxic effect of the cry proteins on the insect midgut (40). *B. thuringiensis* is regarded as a "biorational" control agent because it is non-toxic to vertebrates and non-target insects. However, a problem with commercial *Bt* preparations is their limited field stability. Also, timing of application is critical because susceptibility to *Bt* decreases as larvae mature.

The development of insect-resistant soybean cultivars is also a potential control strategy. The expression of *Bt cry* genes in soybean provides an alternative technology that could avoid the problems associated with field application of commercial formulations. Native *Bt* genes can express *Bt cry* protein in transgenic plants. Although low, these expression levels are still able to suppress feeding by highly susceptible insects. Thus far, a polypeptide from *B. thuringiensis* var. *berliner* suppressed feeding by tobacco hornworm in transgenic tobacco (52). A truncated *B. thuringiensis* var. *kurstaki* HD-1 construct in tomato controlled tobacco hornworm (21,26) and, to a lesser extent, tomato pinworm and tomato fruitworm (21). Other HD-1 constructs were effective against tobacco hornworm in tobacco (5,54), but not (8) or only slightly effective (9) against tobacco budworm on cotton. An HD-73 construct suppressed feeding on potato (15) and tobacco (30). The level of resistance has been increased in plants of cotton (47,55), potato (1), and maize (37) through the use of *Bt* constructs optimized for expression in plants.

Insects can become resistant to *Bt* proteins, a problem that might be overcome by combining *Bt* genes with other host plant resistance factors in soybean (44). Toward that end, this paper describes the engineering of a *Bt cryIA(b)* gene into soybean and compares the insecticidal activity of T_1 soybean plants producing low levels of *Bt* protein with that of a breeding line that has resistance to insect defoliation introgressed into it.

MATERIALS AND METHODS

Plant material. Three soybean genotypes were selected for transformation. The first was "Williams 82", a widely grown cultivar, and the

second was MB80–281, a breeding line derived from "Union" × ["Williams" × PI171451]. This line was selected because it is partially resistant to defoliating insects, and was provided by Dr. R. L. Bernard, USDA-ARS, University of Illinois, Urbana, IL. The third genotype was F376, a breeding line derived from a cross of "Peking" × "Masshokutou (kou 502)". F376 was selected because of its rapid growth in embryogenic suspensions. Peking had performed well in our culture protocol, whereas Masshokutou (kou 502) had been previously identified as a genotype with an outstanding embryogenic capacity and high conversion frequency (4,36).

Culture protocol. Pods were harvested from greenhouse-grown plants when seeds were 4- to 5-mm long. Pods were surface-sterilized by immersion in 70% 2-propanol for 30 s, followed by 12 min in 1% NaOCl (20% commercial bleach, vol/vol), and three rinses in sterile water. The zygotic embryos were excised from the immature seed, the embryonic axes removed, and the cotyledons placed, adaxial side up, on MSD40 medium consisting of MS salts (45) supplemented with B5 vitamins (27), 6% sucrose, 40 mg/liter of 2,4-dichlorophenoxyacetic acid (2,4-D), pH adjusted to 7.0, solidified with 3 *g*/liter of Gelrite (Chemical Dynamics Corporation, South Plainfield, NJ), and dispensed into 100 × 20-mm disposable plastic petri plates. Each plate contained 20 cotyledons. Cultures were sealed with Nescofilm (Karlan Research Products Corp., Santa Rosa, CA) and maintained at 25° C under a 23-h photoperiod provided by cool white fluorescent bulbs. Light intensity averaged 75 to 100 μM photons $\cdot m^{-2} \cdot s^{-1}$.

After 45 days, globular-stage somatic embryos were transferred to liquid Finer and Nagasawa (24) medium in 125-ml triple-baffle Erlenmeyer flasks and maintained on a rotary shaker at 125 rpm under continuous illumination. Otherwise, ambient conditions were as previously described. Cultures were transferred to fresh medium at biweekly intervals.

Gene construct. A truncated *B. thuringiensis cryIA(b)* insecticidal protein gene was used. The isolation and sequence of this gene has been described by Adang et al. (2,3). This gene is highly specific for certain lepidopteran species and has insecticidal activity against soybean and cabbage loopers. The construct consists of a 1.7 kb fragment from the 5′ region of the gene, the cauliflower mosaic virus 35S promoter with an alfalfa mosaic virus leader sequence to enhance its expression and uses the *orf25* polyadenylation sequence. This expression cassette has BglII sites at each end, permitting it to be inserted into the BglII site of pH602, to create pH625. The pH602 vector was developed as a "micro Ti" binary vector for use in *Agrobacterium tumefaciens*-mediated gene transfer. It is based on pH575 (25) but allows for selection of transformed plant cells on medium containing hygromycin instead of kanamycin. This was accomplished by replacing the kanamycin resistance expression unit of pH575 with the *hph* coding sequence driven by the cauliflower mosaic virus 35S promoter and the T-DNA *Orf25/26* polyadenylation sequence (46).

Transformation and selection. Before microprojectile bombardment, 150 embryogenic clumps each of Williams 82 and MB80-281, and 195 of F376, ranging in size from 1.5 to 2 mm in diameter were removed from liquid medium and placed in the center of petri dishes containing MSD40 medium. Each plate was covered with a sterile 20 × 20 mesh of 016 wire (Newark Wire Cloth Company, Newark, NJ), and placed in a DuPont Biolistic PDS-1000 particle gun chamber for bombardment.

Escherichia coli strain MC1061 (pH625) was grown overnight in LB broth (41) supplemented with 50 μg/ml kanamycin and 200 μg/ml erythromycin. The plasmid was extracted using the standard alkaline lysis procedure(41). Plasmid DNA was precipitated onto 1.1-μm tungsten particles using $CaCl_2$/spermidine method of Gordon-Kamm et al. (28). Each plate from each genotype was bombarded once. After bombardment, the clumps were returned to Finer and Nagasawa medium for a month, after which the suspensions were transferred to fresh medium supplemented with 50 mg/liter of hygromycin. After 4 wk, a clump with a green sector was identified in genotype F376 and transferred to fresh medium with hygromycin. All other material was discarded. The green sector was initially divided into three cell lines, each of which was propagated separately, being subcultured at biweekly intervals until enough material was available to begin the plant regeneration process.

Regeneration of plants. Plants were recovered using the procedures described by Bailey et al. (4). Embryogenic tissues were removed from liquid culture and placed on MSM6AC medium, a growth-regulator-free medium consisting of MS salts, B5 vitamins, 6% maltose, 0.5% activated charcoal, pH 5.8, and solidified with 3 *g*/liter Gelrite. After 3 wk, the somatic embryos were separated from the clumps and transferred individually to MSO medium (MS salts, B5 vitamins, 3% sucrose, pH 5.8, 3 *g*/liter Gelrite) for a 4-wk maturation period. Somatic embryos were desiccated for 1 wk in a dry petri dish sealed with Nescofilm. Germination occurred once embryos were returned to MSO medium. Germinating embryos were transferred to GA-7 vessels (Magenta Corp., Chicago, IL) containing MSO medium. Once the root and shoot systems were well developed, the seedlings were transferred to 5-cm pots containing equal portions (vol/vol) of sand and Hyponex potting soil (Hyponex Corp., Maryville, OH) and placed inside two GA-7 vessels connected with a coupler. After a 3- to 5-day establishment period, the top vessel was removed to permit the seedlings to acclimate. Seedlings were transferred to a greenhouse and placed under a 16-h photoperiod provided by high-intensity metal halide lamps. The photoperiod was reduced to 14 h to induce flowering once the plants were large enough. Seed were harvested from mature plants.

Southern hybridization analysis. Young leaf tissue was harvested from T_0 plants, frozen in liquid nitrogen, and lyophilized overnight. One gram of lyophilized tissue was ground into a powder, and DNA extracted using the procedure of Keim et al. (34). Approximately 10 μg of DNA per sample were digested with a HindIII or BglII. Two nanograms of plasmid DNA was added to the final lane. Blotting and hybridization was as described by McCouch et al. (43), except that the hybridization buffer was modified to 1 *M* NaCl, 1% sodium dodecyl sulfate, and 50 m*M* tris pH 7.5. A portion of the *Bt* coding sequence from pH626 was amplified by polymerase chain reaction (PCR) using primers of sequences 5′ TTTGTTCCCGGTGCTGG and 5′-GTGGTAGAAGCGTAGCG, representing nucleotides 148–164 of the *Bt* sequence for the forward primer, and nucleotides 1598–1582 for the reverse primer. The probe was labeled using random primers.

Hygromycin resistance assays. Hygromycin resistance assays were conducted to determine the presence of *hpn* activity in plant tissues of T_0 and T_1 plants. Leaf and petiole tissue samples were collected, surface-sterilized as described previously, and placed on MSDK medium, consisting of MS salts, B5 vitamins, 3% sucrose, 2 *g*/liter Gelrite, 1 mg/liter 2,4-D, and 0.2 mg/liter kinetin (56). After 2 wk, resulting callus was transferred to MSDK medium supplemented with 100 mg/liter hygromycin.

Chromosome counts. Root tips were collected from T_1 plants, pretreated with 3 m*M* 8-hydroxyquinoline for 6 h at 4° C, fixed overnight in 3:1 100% ethanol:glacial acetic acid (vol/vol), hydrolyzed in 2:1 concentrated hydrochloric acid:70% ethanol (vol/vol) for 5 s, and squashed in carbol fuchsin as modified by Kao (33).

Enzyme-linked immunoassay (ELISA) assays. Quantitative analyses of *Bt* cry protein produced in soybean were done using a double antibody sandwich ELISA as described in Adang et al. (1).

Insect bioassays. Two T_0 plants from cell lines 5 and 7 were selected based on ELISA assays, and 12 T_1 plants from each one were used for further evaluations. Trifoliolate leaves from each plant were detached from the plants, and the petioles placed in a vial of water that in turn was placed in plastic containers 15.24 cm in diameter × 6.35 cm in depth, using one leaf per container. A wire mesh was placed over the water vial to provide support for the leaf. Eggs of velvetbean caterpillar (*Anticarsia gemnalis* Hubner) were obtained from the Southern Field Crop Management Laboratory, Stoneville, MS. Fifteen neonate larvae were placed on each trifoliolate leaf, and allowed to feed for 5 days. Controls included F376 plants which were non-transgenic but had passed through the entire regeneration protocol, "Cobb" (a cultivar susceptible to defoliation), and GatIR81–296, a breeding line with inherent insect antibiosis (7,48). At the end of the feeding period, containers were opened and the number of dead and live larvae counted. Live larvae were killed in a freezer, weighed, and measured with a micrometer under a dissecting microscope. The length and head capsule width of each larvae were measured, with length and head capsule width being a measure of larval health and developmental stage, respectively. Visual estimates of percent defoliation were also made. This procedure was repeated 3 times for each plant.

Polymerase chain reaction analysis. Leaf tissue remaining from the insect assays was pressed and dried. DNA was isolated from these leaves using a CTAB miniprep (51). The only deviations from the published protocol were the maceration of 15 mg dried tissue, and the inclusion of a 10 min, 70° C incubation step after maceration. Polymerase chain reaction analysis was performed to verify the presence of the transgene in the leaf tissue. Because the primers described earlier were found to amplify a band

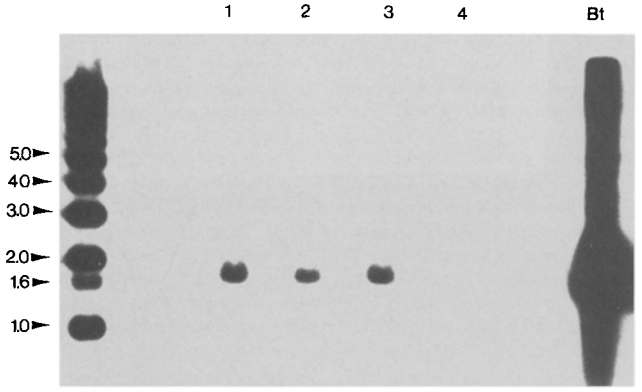

FIG. 1. *Lane 1* = kb ladder; *lanes 1–3* = HindIII digest of DNA from plants obtained from 3 transgenic cell lines; *lane 4* = control; *Bt* = HindIII truncated *cryIA(b)* sequence.

about the expected size of the transgene in all non-transgenic soybean genotypes tested, a new set of primers was designed using version 0.5 of Primer software (MIT Center for Genome Research and Whitehead Institute for Biomedical Research, Cambridge, MA). The sequences of these were 5′-CAGAAAAAGCGGAACGGTAG for the forward primer and 5′-TCCTCCTGTAAATCCTGGTCC for the reverse primer, representing nucleotides 1203–1222 and 1491–1471 of the *Bt* sequence. The PCR was performed in 50 μl total volume, containing 50 m*M* KCl, 10 m*M* Tris-HCl pH 8.3, 2.0 m*M* $MgCl_2$, 0.1 m*M* of each deoxynucleotide, 50 pmol of each primer, 1.75 U *Taq* DNA polymerase (Promega), 2% vol/vol glycerol, and 50 ng template DNA. Reactions were overlaid with 50 μl light mineral oil and denatured for 5 min at 95° C. Reactions were cycled 40 times through 95° C for 10 s, 60° C for 20 s, 72° C for 60 s using a Perkin-Elmer DNA Thermal Cycler. The amplification products (15 μl) were electrophoresed at 4 V/cm using a composite gel consisting of 1% Synergel (Newton Centre, CT) and 0.8% agarose. The products were visualized by ethidium bromide staining.

RESULTS AND DISCUSSION

Although soybean transformation is not routine, various techniques have been used to obtain transgenic plants. These techniques include cocultivation of germinating seeds (14) or seedling cotyledons (13,29,57) with *Agrobacterium tumefaciens*, bombardment of embryonic meristems (16–20,42) or embryogenic suspensions (23) with microprojectiles, and microinjection of ovules (38,39). None of these have been noted for their efficiency.

In this study, embryogenic suspension cultures were established from each of the three soybean genotypes evaluated, but a transgenic cell line was only obtained from F376, a result that may be due to differences in growth rate and culture morphology between the three genotypes. Embryos of F376 growing in suspension were also smaller and more numerous than those of the other genotypes. Even so, the frequency of transformation (one cell line from 195 clumps in the case of F376, none in the case of the others) remains extremely low.

The transformed cell line was initially separated and propagated as three cell lines, and plants were regenerated from each of these three secondary lines. A total of 40 plants from each of two of these cell lines, labeled 5 and 7, were recovered. Most of these plants were at least partially fertile and set seed. The third cell line was not prolific, grew slowly, and yielded only 10 sterile plants. Partial sterility may be related to chromosomal abnormalities acquired during the transformation procedure. Soybean normally has 2n = 40 chromosomes, but all the transgenic plants recovered were aneuploids, with an additional 5–10 chromosomes and lowered seed set. Fertility did not improve in subsequent generations, and some progeny were completely sterile. In contrast, all plants of F376 regenerated from nontransgenic cell lines had normal seed set.

Callus from all T_0 plants grew normally on medium supplemented with 100 mg/liter of hygromycin, and Southern hybridization analysis of these plants revealed the expected 1.7 kb HindIII fragment (Fig. 1). However, a BglII digest of all lines yielded a fragment approximately twice as large as the expected 3.4 kb fragment, suggesting that a BglII site in the cassette was altered in the plasmid. The T_1 plants from both cell lines segregated 3:1 for hygromycin resistance, indicating integration into one locus.

When T_1 plants were subjected to feeding by neonate velvetbean caterpillar larvae, hygromycin-tolerant plants derived from cell line 7 deterred larval growth, development, and feeding, whereas hygromycin-sensitive plants of the same cell line did not (Table 1, Fig. 2). The level of feeding deterrence conferred by the transgene was comparable to that exhibited by GatIR81–296, a breeding line with

TABLE 1

FEEDING AND DEVELOPMENT OF VELVETBEAN CATERPILLAR LARVAE ON TRANSGENIC AND NONTRANSGENIC SOYBEAN PLANTS DERIVED FROM TWO CELL LINES, AND SOYBEAN GENOTYPES WITH LOW (COBB) AND MODERATE (GatIR81-296) NATURAL RESISTANCE TO NOCTUID DEFOLIATORS

Genotype or Cell Line[a]	Number of Plants	Percent Defoliation	Mean No. Surviving Larvae[b]	Mean Larval Weight[c]	Mean Larval Length[d]	Mean Capsule Width[d]
5+	9	44.8 ± 5.1[e]	9.3 ± 0.7[e]	8.3 ± 0.8[e]	9.5 ± 2.4[e]	0.8 ± 0.1[e]
5−	3	37.1 ± 8.8	6.7 ± 1.3	6.4 ± 1.3	9.4 ± 4.2	0.8 ± 0.1
7+	9	19.9 ± 5.1	5.8 ± 0.7	3.9 ± 0.8	7.2 ± 2.4	0.6 ± 0.1
7−	3	44.0 ± 8.8	6.9 ± 1.3	7.9 ± 1.3	10.0 ± 4.2	0.9 ± 0.1
Cobb	5	44.8 ± 6.8	7.7 ± 1.0	6.4 ± 1.0	8.9 ± 3.2	0.7 ± 0.1
F376	5	40.6 ± 6.8	6.8 ± 1.0	5.4 ± 1.0	8.8 ± 3.2	0.8 ± 0.1
GatIR81-296	5	21.7 ± 6.8	4.0 ± 1.0	3.4 ± 1.0	7.5 ± 3.2	0.7 ± 0.1

[a] A "−" sign indicates R_1 plants that lost the transgene through Mendelan segregation, as determined by hygromycin sensitivity. [a] A + sign indicates R_1 plants with at least one copy of the transgene, as determined by hygromycin resistance. [b] Out of 15; [c] In milligrams; [d] In millimeters; [e] Mean ± standard error.

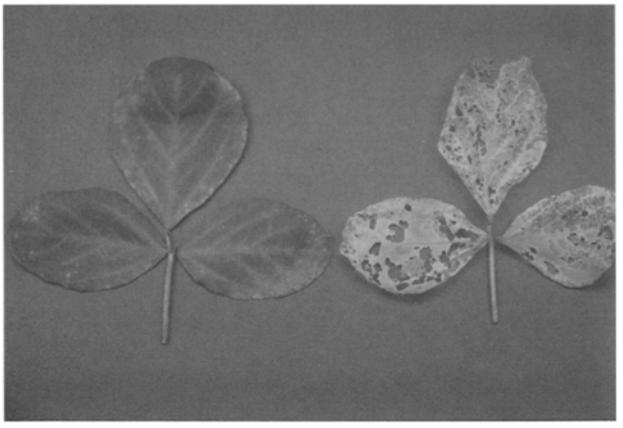

FIG. 2. Transgenic (*left*) and non-transgenic (*right*) leaves of T_1 plants regenerated from cell line 7 and exposed to neonate velvetbean caterpillar larvae for 5 days.

moderate resistance to defoliation (6). Analysis of T_1 leaf samples with PCR revealed the expected 0.29 kb band in the resistant leaves and its absence in the susceptible ones (Fig. 3).

Adang et al. (2) described gene expression and insect resistance of transgenic tomato plants harboring the same construct used in this study, and found toxin levels of 2 to 10 ng/mg of soluble protein from leaf tissues, slightly higher than those detected in these T_0 soybean plants, which ranged from undetectable to 1 to 2 ng/mg of soluble protein. No *Bt* protein was detected in T_1 plants. This was not unexpected, as native *Bt* sequences are not expressed well in plants, given they contain regulatory sequences, require tRNAs not commonly found in plants, or lead to secondary mRNA structures (47). Levels of *Bt* protein too low to detect by ELISA have also been reported in other transgenic plants (26), which nevertheless deterred feeding by caterpillars. Similarly, levels of transgene mRNA too low to detect with conventional Northern analysis have been reported (15), as have *Bt* protein levels ranging from 1 ng/mg (12,21) to 3 ng/mg of soluble protein (52), with concentrations one-fifth as high still being effective for control of tobacco hornworm. Concentrations ranging from 0.0042 to 0.061% of total extracted protein help control tobacco budworm on cotton (32).

The single transformation origin of all plants from this study was verified by Southern analysis using genomic DNA digested with BamHI, an enzyme with a unique restriction site in the plasmid used for transformation. Plants from all cell lines gave an identical band. It is evident from the hygromycin resistance of callus tissue and the PCR analysis of the T_1 plants that transgene integration is stable. However, expression of the transgene and the overall phenotype of the plants was not stable. Although all plants obtained came from a single transformation event, the original cell line was divided at an early stage into three separate cell lines. Plants from each of these cell lines had distinct phenotypes. One cell line grew slowly, converted into plants with difficulty, and gave completely sterile plants. Of the remaining two lines, one failed to have insecticidal activity. It is difficult to attribute these phenotypic differences to instability of the transgenic DNA, as the transgenic gene seems stable. One possible explanation for the lack of *Bt* effectiveness in one cell line could be silencing of the transgene, a phenomenon that is well documented in the literature (e.g., 11,35,49). Another explanation for these differences is that somaclonal variation accumulated by these cell lines after they were separated affected insect resistance in the susceptible line. Finally, it is possible that somaclonal variation was the source of resistance in the resistant line. As insect resistance in PI229358, the source of resistance in GatIR81–296, is conditioned by an estimated four genes (50), this latter possibility is unlikely because changes in several genes would be necessary to obtain the level of resistance measured in this study. Thus far, single genes with a large deterrent effect on insect feeding are not known in soybean.

Combining *Bt* genes with other unrelated transgenes or with other soybean genes that confer insect resistance, such as those from GatIR81–296, may be a necessary strategy to prevent the development of new insect biotypes that can overcome host resistance conditioned by a single inhibitory factor. Various sources of resistance could be pyramided in crop cultivars, or deployed through the use of crop rotations or mosaic plantings (44). Such a strategy would be analogous to that proposed by Brattsten (10), who

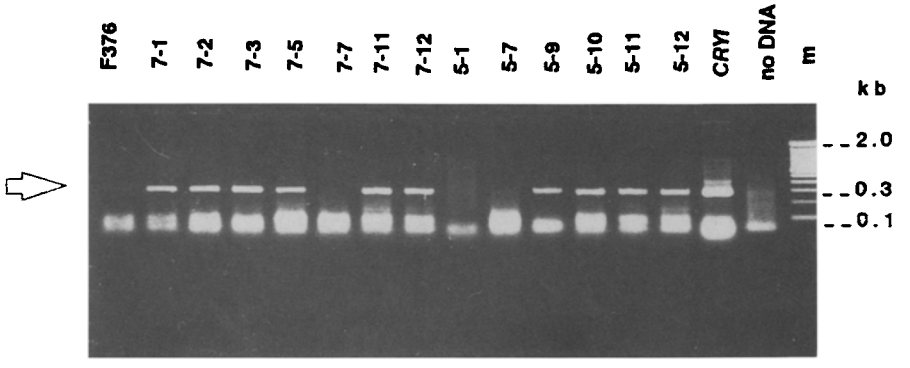

FIG. 3. *Bt CryIA(b)* 289 bp PCR products in T_1 plants. *Lanes* labeled 7-X and 5-X are the T_1 transgenic soybean plants evaluated for insect resistance. *Lanes "m"* are 100 bp DNA marker (GIBCO-BRL, Gaithersburg, MD). *Lane "F376"* is the parental genotype (untransformed). *Lane "CryI"* is pH625, the *Bt CryIA(b)*-containing plasmid, which served as a PCR positive control. *Lane "no DNA"* is the PCR negative control (template = water). Plants without the *Bt* band were invariably sensitive to hygromycin, and if from cell line 7, were also sensitive to insect feeding.

advocated using plants that produce several different compounds that confer pest resistance in plants. Likewise, Van Rie (53) suggested combining different types of *Bt* genes together to achieve a more lasting resistance.

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