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Bacillus thuringiensis δ-Endotoxin Expressed in Transgenic *Nicotiana tabacum* Provides Resistance to Lepidopteran Insects

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ABSTRACT

The crystal proteins, or δ-endotoxins, of *Bacillus thuringiensis* are specifically lethal to Lepidopteran insects. We utilized a truncated and modified portion of a cloned crystal protein gene to construct a chimeric gene capable of expression in plant cells. Using an *Agrobacterium tumefaciens* binary vector system, we then transferred the chimeric toxin gene into tobacco (*Nicotiana tabacum* cv Havana 425) cells and regenerated recombinant plants. One to several copies per cell of the toxin gene are routinely present in the recombinant plants. Hybridization experiments demonstrated that these plants had a new RNA species of the size expected for the truncated toxin mRNA, and a polypeptide having the mobility expected for the truncated toxin was detected by immunoblotting. Significant variation was found in the levels of toxin-specific RNA expression between different recombinants, but the levels of hybridizing RNA in transformants correlated with the level of toxicity demonstrated against *Manduca sexta* (tobacco hornworm), and other Lepidopteran insects. The recombinant genes were transmitted to progeny and resistance to insects was maintained, thus demonstrating that the introduction of toxin genes into plants may be a practical method of providing protection against certain insect pests.

Bacillus thuringiensis is a bacterium that forms insecticidal proteinaceous crystals during sporulation (2, 21, 28). The crystal protein, representing as much as 30% of the spore dry weight, consists predominately of one or more protoxin species of up to 160,000 D. The protoxins can be cleaved by proteolysis to peptides of 55,000 to 70,000 D that are specifically toxic to Lepidopteran, Dipteran, or Coleopteran insects (2, 21, 28). Deletion analyses have localized the toxic portion of the protoxins to the amino-termini and have shown that both amino- and carboxyl-terminal fusions can be made to the toxin without loss of insecticidal activity (23). While the precise mode of action of the toxin is not yet known, ingestion of toxin by susceptible insects results first in a paralysis of the gut and mouth parts, followed by a reduction or cessation of feeding, a gradual deterioration of the gut epithelial cells, and finally death of the organism (2).

Microbial formulations of *B.t.* toxins[1] have been applied to an increasing variety of agricultural crops for over 20 years, with current worldwide annual use in excess of 2.3 million kg (21). Practical limitations such as poor persistence (25) and the relatively high costs incurred by multiple applications have limited product acceptance. However, major insect pests are developing resistance to most classes of chemical insecticides, and environmental damage caused by insecticidal chemicals is an increasing public concern. The high specificity of the *B.t.* toxins make them an attractive alternative to petrochemicals if efficiency of use can be improved. For this reason, we and others (10, 27) have explored the feasibility of generating Lepidopteran-resistant transgenic plants. This report presents data on the development of transgenic tobacco plants that produce sufficient levels of *B.t.* toxin to either kill Lepidopteran insects or inhibit their feeding. Analysis of the expression of the chimeric gene in the resistant plants has revealed several problems which affect the levels of toxin gene expression. These problems must now be resolved in order to obtain additional insect resistant agricultural crops.

MATERIALS AND METHODS

Source of Materials. Seeds of tobacco (*Nicotiana tabacum* cv Havana 425) were surface sterilized (26) and germinated on Murashige and Skoog medium (18); aseptically grown leaves and stems were used for transformations, described below. *Agrobacterium tumefaciens* strain EHA101 (15) was obtained from E. Hood. *A. tumefaciens* binary vector components have been described previously (26). DNA encoding a δ-endotoxin from *B. thuringiensis* var kurstaki HD-1-Dipel (specific for Lepidopteran insects) was obtained from plasmid pES1 (24). Eggs of *Manduca sexta* (tobacco hornworm) were purchased from Carolina Biological Supply. Eggs of *Heliothis virescens* (cotton bollworm), *Heliothis zea* (corn earworm), and *Spodoptera exigua* (beet armyworm) were purchased from the USDA Southern Regional Research Laboratory. Enzymes utilized in recombinant manipulations were purchased from New England Biolabs (Beverly, MA) and used as directed, unless otherwise noted.

pAMVBTS Construction. The plasmid AMVBTS consists of the ampicillin-resistant vector pCMC66 (26) containing a chimeric truncated *B.t.* toxin gene. The complexity of the chimeric gene required that various intermediate plasmids be constructed prior to completion of pAMVBTS, with the various components then ligated together in the final plasmid. The transcriptional promoter on the chimeric *B.t.* gene corresponds to nucleotides 7013 to 7440 of the CaMV sequence (12), which comprises the 35S promoter and enhancer region (19). This fragment was excised from pCaMV10 (14), with the ends of the fragment modified by addition of commercial linkers. The 5′ end of the promoter fragment, originally a blunt *Hin*cII restriction site, was converted to *Xho*I by ligation directly to commercial linkers. The 3′ end of the promoter fragment, originally an *Hph*I site, was first blunted by reaction with Klenow polymerase, then ligated to commercial *Hin*dIII linkers. The sequence of the region near the new *Hin*dIII site is displayed in Figure 1, with the *Hin*dIII site located at nucleotide +4 relative to the predicted start of transcription (19). The 5′ untranslated region of the gene in pAMVBTS (Fig. 1) was generated by synthesis of two comple-

[1] Abbreviations: *B.t.* toxin, *Bacillus thuringiensis* δ-endotoxin; CaMV, cauliflower mosaic virus; AMV, alfalfa mosaic virus; NPT-II, neomycin phosphotransferase-II; kb, kilobase pairs.

mentary oligonucleotides that anneal to form compatible *Hin*dIII and *Nco*I sticky ends, with the internal sequence corresponding to the untranslated region of AMV RNA 4 (13). The *Hin*dIII end of the oligonucleotide was ligated to the CaMV promoter fragment, and the *Nco*I end was ligated to the 5′ terminus of the *B.t.* toxin coding sequence.

The truncated *B.t.* toxin coding sequence on pAMVBTS corresponds to nucleotides 527 to 2463 of the sequence (24). The 5′ terminus of the coding region present on pSYC823 (a pUC8 vector containing the subcloned *B.t.* toxin gene found on pES1), was converted to an *Nco*I restriction site, CCATGG, with the use of a polymerase chain reaction and synthetic primers (22). The internal 'ATG' of the *Nco*I site represents the initiation codon of the toxin. The 3′ terminus of the truncated toxin was generated by first cleaving the toxin gene with *Bcl*I at nucleotide 2458 (24), followed by ligation of a synthetic linker to the *Bcl*I site. The sequence of the linker, formed by annealing two homologous oligonucleotides that generated *Bcl*I and *Pst*I ends, is noted in Figure 1. The linker added two additional proline codons to the toxin, then terminated the peptide with two adjacent termination codons. The *Pst*I end of the termination linker was ligated to a DNA fragment encoding the polyadenylation region of nopaline synthase as described previously (26).

Construction of Agrobacterium Vector pTV4AMVBTS. The components of the *A. tumefaciens* binary vector system used in these studies were similar to those described by Umbeck *et al.* (26), except that pTV4 was substituted for pCMC92 as the initial carrier plasmid and *A. tumefaciens* strain EHA101 (15) served as the transfer host. The vector pTV4 (Fig. 1) is a derivative of pCMC92 (26) in which the region of Ti plasmid DNA present on pCMC92 5′ to the nopaline synthase promoter has been deleted, and replaced on pTV4 with synthetic DNA consisting of the Ti plasmid right border of pTiT37 (29) and a consensus overdrive (20) derived from plasmids pTiT37 (9) and pTiA6NC (20).

Plasmid AMVBTS was co-integrated into pTV4 by ligation at unique *Xho*I restriction sites on each plasmid. The orientation as shown (Fig. 1) places the CaMV 35S enhancer (19) on the pAMVBTS 35S promoter adjacent to the nopaline synthase promoter, and thus provides a bidirectional enhancement. The plasmid TV4AMVBTS was conjugated into *A. tumefaciens* strain EHA101 (15) as previously described (3).

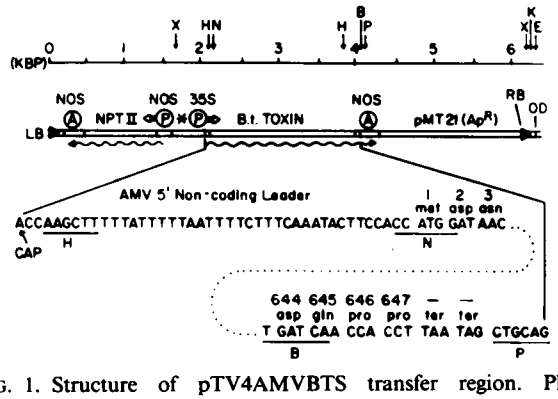

FIG. 1. Structure of pTV4AMVBTS transfer region. Plasmid TV4AMVBTS is a 16 kb wide-host-range component of an *A. tumefaciens* binary vector system (26), formed by co-integration at unique *Xho*I sites (shown) of 4.6 kb pAMVBTS and 11.6 kb pTV4. Displayed is the T-DNA region with left (LB) and right (RB) T-DNA borders of pTiT37 (29) bracketing chimeric genes encoding a selectable marker (NPT-II) and a truncated *B.t.* toxin. Scale (top line) is kilobase pairs (KBP). Abbreviations: Nos A and Nos P, nopaline synthase polyadenylation region and promoter, respectively (9); 35S P, CaMV 35S promoter (19); pMT21(ApR), ampicillin resistant plasmid pMT21; OD, consensus overdrive (20); AMV 5′noncoding leader refers to untranslated region of AMV RNA 4 (13); toxin amino- and carboxy-terminal codons are noted and numbered; * is the 35S enhancer. Restriction enzymes (underlined): *Bcl*I (B), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Nco*I (N), *Pst*I (P); *Xho*I (X).

Plant Transformations. Aseptically grown immature stems and leaves were inoculated with overnight cultures of *A. tumefaciens* (26). Following 48 to 72 h of incubation at room temperature on regeneration medium (MS medium [18] plus 1 mg/ml kinetin), cefotaxime (100 μg/ml) and vancomycin (250 μg/ml) were applied to kill the agrobacteria, and kanamycin (100 μg/ml) was applied to select transformed plant tissues. After approximately 6 weeks with media changes at 2-week intervals, shoots appeared. Shoots were excised and placed on rooting medium (3) plus 25 mg/L kanamycin until roots had formed (1–3 weeks), after which time plants were transferred to commercial soil mixture (Metro-Mix 360, W. R. Grace & Co.). Approximately 2 weeks after potting, insect toxicity tests were initiated.

Insect Toxicity Assays. Insect eggs were hatched on mature wild-type tobacco plants, and larvae were allowed to graze for 1 to 3 d prior to transfer to test plants. Mature tobacco plants contain higher levels of secondary metabolites than freshly regenerated plants, so larvae with feeding exposure to older plants were considerably less sensitive to *B.t.* toxin than neonatal larvae. The decreased sensitivity proved useful in distinguishing between variations in toxin levels in transgenic plants. Tobacco hornworms were placed directly on leaves of young wild-type and recombinant plants, usually two to four larvae per plant per test with up to six successive tests conducted per plant. Only test plants showing 100% toxicity in all tests were considered to be resistant. Alternatively, tests were conducted using excised leaf tissue in Petri dishes, with 5 to 10 hornworms or a single larva of other species per dish. In dish assays, weights of larvae were recorded at inititation and termination of tests. Feeding trials were generally 2 to 4 d in duration, with daily monitoring of reductions in feeding and larval deaths.

Southern Blot Analysis. Total nucleic acid was prepared from young plant tissues as described by Dellaporta (8). Ten μg DNA, estimated by agarose gel staining and fluorimetry, was digested with appropriate restriction enzymes and samples were then electrophoresed on 0.6% agarose. DNA was transferred to nylon membranes (Biodyne membranes, Pall), and hybridizations were conducted using [^{32}P]RNA probes as previously described (26). Filters were autoradiographed on X-AR5 film at −80°C with two intensifying screens (Cronex Lightning Plus, DuPont).

RNA Hybridizations. RNA standard transcripts for purposes of size and concentration comparison were synthesized using Riboprobe transcription kits (Promega Biotec). Templates were either NPT-II or truncated *B.t.* toxin coding regions of defined length and [^{3}H]CTP (Amersham) was incorporated at known specific activity for accurate concentration determination. Total RNA was isolated from plant leaves as described by Chirgwin *et al.* (7), except that the concentration of guanidinium thiocyanate was increased to 5 M. Following resuspension of RNA pellets after CsCl centrifugation, 10 to 40 μg RNA were either prepared for slot-blot hybridizations as recommended by the supplier of the apparatus (Schleicher & Schuell) or electrophoresed on formaldehyde-containing agarose gels as described by John *et al.* (16). After electrophoresis, gels were blotted onto nitrocellulose for hybridizations (16). [α-^{32}P]dCTP-DNA oligonucleotides were synthesized as probes for hybridizations as described for the Oligolabeling Kit (Pharmacia). Hybridizations were conducted overnight at 42°C in buffer (50 mM Tris-HCl, 3 mM Na-pyrophosphate, 1% SDS, 5 mM EDTA [pH 7.5]) containing 50% formamide, 5× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate), 150 μg/ml salmon sperm DNA, and 10× Denhardts' solution (0.2% BSA, 0.2% Ficoll, 0.2% PVP). Filters were extensively washed, with a final wash in 0.1× SSC at 60°C. Hybridization signals were detected by autoradiography for 1 to 3 d at

Table I. *Toxicity in Regenerated AMVBTS Tobacco Plants*

Kanamycin-resistant plants were regenerated following transformation with pTV4AMVBTS. The number of inserted copies of AMVBTS genes were determined by restriction mapping and Southern hybridizations, the level of toxin-related RNA (pg/20 μg) in each plant was determined (see Fig. 4A and "Materials and Methods"), and toxicity of plants to *M. sexta* larvae was evaluated. Little or no toxicity is scored (−); (+) to (++++) refer to increasing reductions in feeding leading to complete larval mortality. The ratio in 'Toxicity' refers to (no. larvae killed/no. tested); H425 refers to wild-type tobacco.

Plant No.	Genes	RNA	Toxicity
H425	0	ND[a]	− (0/50)
857	3	47	++++ (12/12)
858	0	1.2	− (1/6)
859	2	1.1	+++ (10/10)
860	1	0.8	+++ (8/8)
861	2	1.4	++ (8/8)
862	5	7	++++ (8/8)
863	1	0.5	+ (6/6)
870	3	2.5	+++ (10/10)
872	3	1.3	++ (8/8)
884	0	2.8	− (2/6)

[a] Not detectable.

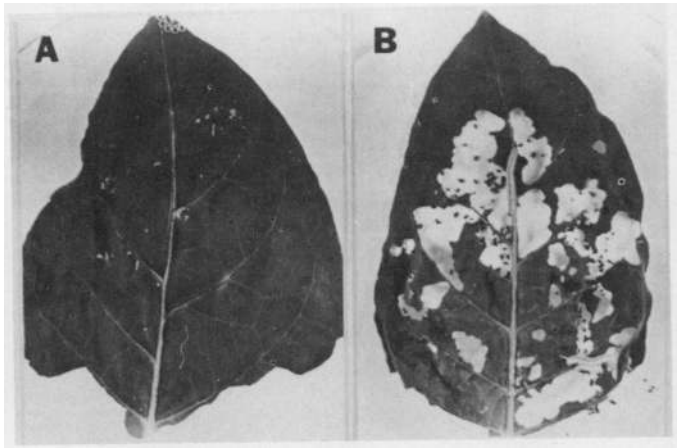

FIG. 2. Bioassay of AMVBTS transformant No. 857. Leaves of plant No. 1274 (A) and untransformed H425 (B) were placed in 10 cm Petri dishes, and five larvae of *Manduca sexta* were applied. Incubation was carried out at 25° C in the dark for 48 h, with monitoring of feeding reduction and mortality recorded at 24 and 48 h. All five larvae feeding on plant No. 1274 were dead at 24 h; the larvae on H425 leaves continued to feed until termination of the test.

−80°C using X-AR5 film with two intensifying screens (Cronex Lightning Plus, DuPont).

Protein Extraction and Immunoblots. Production of truncated *B.t.* toxin for use in insect feeding trials and as standards on immunoblots was accomplished by substituting the *tac* promoter on pKK233-2 (1) for the CaMV 35S promoter on pAMVBTS, and growing the host *E. coli* under conditions which provide for *tac* promoter induction (1). For extraction of toxin from recombinant plants, young leaf tissue was frozen in liquid N_2, powdered, then sonicated briefly in an equivalent (w/v) amount of extraction buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5% NP40, 1% 2-mercaptoethanol, 4% SDS, 2.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml leupeptin, and 50 μg/ml each of antipain, pepstatin, and aprotinin, final pH 12.5). Immediately following sonication, extracts were boiled and supernatants were collected following microcentrifugation. Acrylamide gel electrophoresis of supernatants and toxin standards was conducted, proteins were blotted onto nitrocellulose, and enzyme-linked immunochemical staining was carried out (5, 6). Briefly, nitrocellulose blots were incubated with rabbit anti-*B.t.* toxin serum (1:1000 dilution) and subsequently with alkaline phosphatase-conjugated anti-rabbit immunoglobulin (1:1000 dilution). The immunoblots were stained with 5-bromo-4-chloro-3-indoylyl phosphate (*p*-toluidine salt, Sigma) and *p*-nitro-blue-tetrazolium chloride (Sigma).

RESULTS

***B.t.* Toxin Gene and Vectors.** Isolation and sequence analysis of the δ-endotoxin gene of *B. thuringiensis* var kurstaki HD-1-Dipel has been described (24). Of the 1176 amino acids present in the crystal protein only approximately 600 amino-terminal residues are required for insect toxin activity (23). The function of the remaining carboxy portion of the protoxin, beyond structural considerations in crystal formation, remains unknown. Because the intact protoxin had seen extensive field use in microbial formulations, we initially attempted to express the entire 1176 amino acid toxin in plant cells. Our initial constructions were similar to those detailed here, but contained the entire toxin coding sequence. Following transformation and selections for kanamycin resistant transformants, we obtained tobacco calli that were shown by immunoblots to contain significant levels of intact protoxin (10–50 ng/mg protein). However, all such calli soon became necrotic and died. Any plants we were able to regenerate from our initial experiments were shown by hybridization analysis and immunoblots to contain either broken or inactive toxin genes (data not shown). We eventually concluded that expression of intact δ-endotoxin was lethal to plant cells. In subsequent experiments, including those described in this report, we eliminated the protoxin carboxy terminus and found no evidence that the truncated toxin is deleterious to plant cell viability.

The plasmid AMVBTS is an expression cassette encoding the truncated *B.t.* toxin, under the transcriptional control of the CaMV 35S promoter (Figure 1). Adaptation of the promoter fragment for use in chimeric gene construction deleted the entire 35S mRNA coding region, with the exception of the 5′ cap site and the penultimate nucleotide (19). We, therefore, reconstructed an mRNA 5′ untranslated region for the toxin transcript by synthesizing a synthetic duplex oligonucleotide corresponding in sequence to the 5′ end of the AMV RNA 4 (13). The AMV RNA 4, which encodes the coat protein, is translated efficiently both *in vivo* and *in vitro*, potentially because of characteristics found in the untranslated region of the RNA (13).

The *B.t.* toxin coding region was modified as described in "Materials and Methods" to have an *Nco*I restriction site at the amino terminus, with nucleotides neighboring the first 'ATG' codon chosen to conform to Kozak's consensus for translational initiation in eucaryotes (17). The toxin was truncated after codon 645, which is 3′ to the proteolytic cleavage site that generates the mature toxin in the insect gut (23). The synthetic linker we ligated to the terminus of the truncated toxin gene supplied two adjacent proline codons followed by two termination codons. The prolines, which are both hydrophobic and resistant to various proteases (4), might be expected to help protect the new carboxy terminus of the peptide.

The binary Ti plasmid vector system used in this set of experiments is similar to that previously reported (26). However, modifications have been introduced in the 'carrier plasmid' TV4, the T-DNA border portion of the vector system. The replacement of an authentic Ti plasmid right border region from pTiT37, as found on pCMC92 (26), with the synthetic T-DNA border and overdrive sequence described here is useful because it decreases the plasmid size by approximately 1.5 kb (to 12 kb) and adds convenient unique restriction sites (including *Xho*I) between our selectable marker (NPT-II) and the border region. Placement of the gene of interest in the *Xho*I site between the marker and the

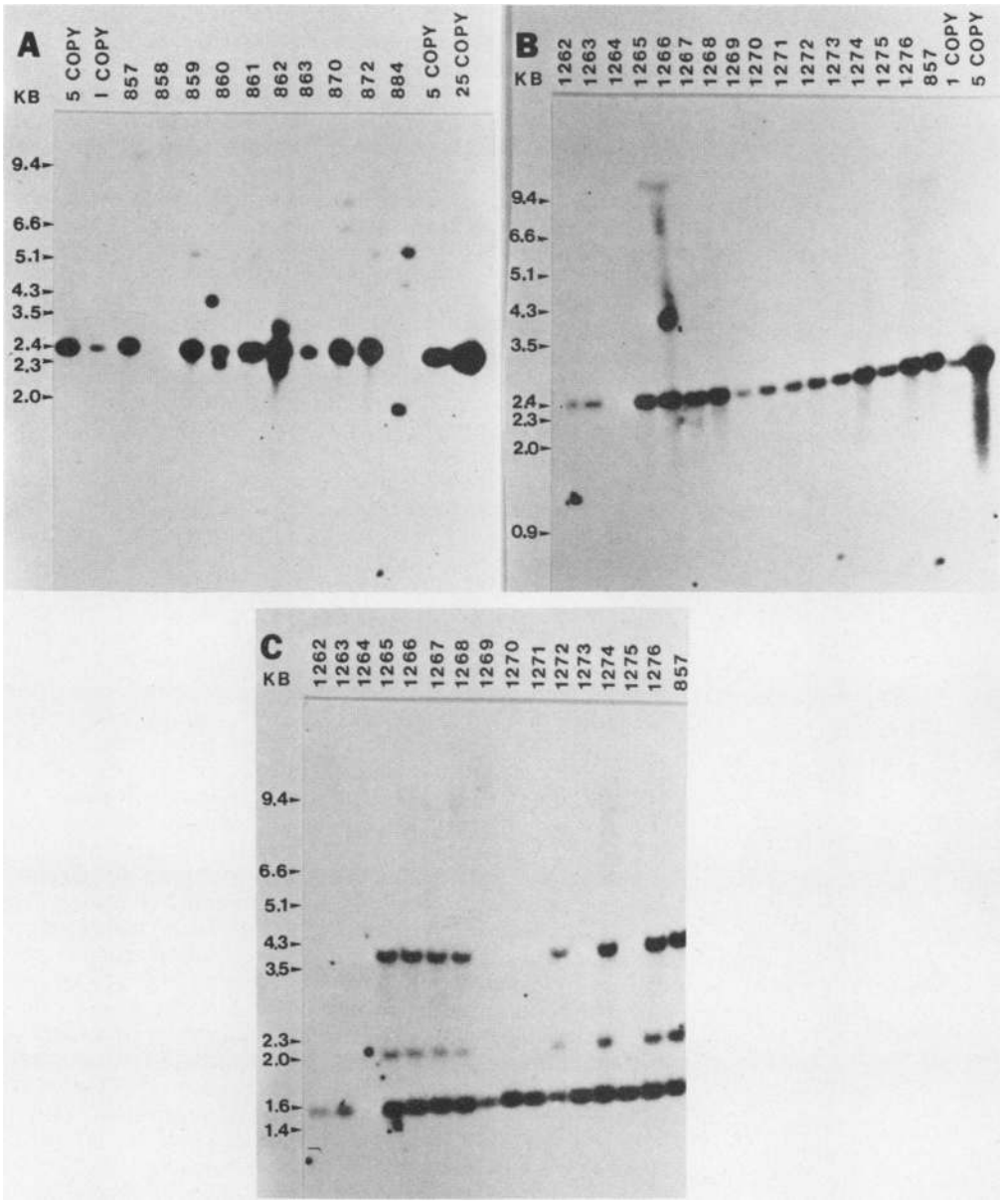

FIG. 3. Southern blot analysis of AMVBTS insertions in regenerated tobacco plants and progeny. Plant DNA (10 μg) was digested with both endonucleases *Xho*I and *Pst*I (A and B) or *Xho*I (C) and electrophoresed on 0.6% agarose gels. DNA was then transferred onto nylon membranes, and hybridized to [^{32}P] RNA probes homologous to the *B.t.* toxin (A and B) or NPT-II (C). DNA copy standards are pTV4AMVBTS plasmid at the calculated concentrations for 1, 5, or 25 insertions per cell combined with 10 μg tobacco DNA. Mobility of standard DNA fragments is noted (KB). A, DNA from regenerated AMVBTS tobacco plants; B and C, DNA from progeny of plant No. 857.

right border generates fewer plant transformants with broken or deleted target genes, since the right border is relatively precise in transfer to plants (29). Use of these synthetic sequences on pTV4 provides no apparent differences in transformation efficiency from our previous plasmids, such as pCMC92 (26).

Plasmid AMVBTS was cointegrated into pTV4 using unique *Xho*I restriction sites on each plasmid. The orientation as shown (Fig. 1) places the CaMV 35S promoter and associated enhancer (19) adjacent to the nopaline synthase promoter (9), and thus is likely to provide a bidirectional enhancement affecting both the NPT-II and toxin genes. This orientation of the two co-integrated plasmids further provides for greater plasmid stability than the opposite orientation, which would result in direct repeats of the nopaline synthase polyadenylation region on the two genes.

Transformation of tobacco tissues with our *A. tumefaciens* vectors resulted in kanamycin-resistant shoots in approximately 6 weeks. The majority of the excised shoots readily formed roots when placed in kanamycin-containing medium. At that time, the young plants were transferred to soil, and growth was continued until completion of insect toxicity tests.

Insect Toxicity. Table I shows the toxicity analysis of one set of 10 AMVBTS transformants. Relative levels of toxicity between those plants providing complete *M. sexta* larvae mortality are subjective, and are based on the extent of damage to plant leaves prior to cessation of feeding. In all cases of mortality, some feeding on test tissues was observed (Fig. 2). Analysis of over 100 independent transformants has shown that approximately 25% of the plants were lethal to all larvae within 4 d, with the more resistant plants allowing only minimal feeding during the early hours of the test. Many plants judged 'nontoxic' (few larvae were killed) did reduce larval feeding levels and growth rates in comparison to control tissues.

Southern Blots. The results of Southern blot analyses on the 10 regenerated AMVBTS plants of Table I are shown in Figure 3A. Digestion of the plant DNA with *Pst*I and *Xho*I should release the toxin chimera as a 2.42 kb internal DNA fragment, which includes the CaMV promoter and the entire toxin coding region (see Fig. 1). Eight of the 10 plants apparently had one or more intact toxin genes, while plants No. 858 and No. 884 showed only broken inserts (hybridizing fragments in plant No.

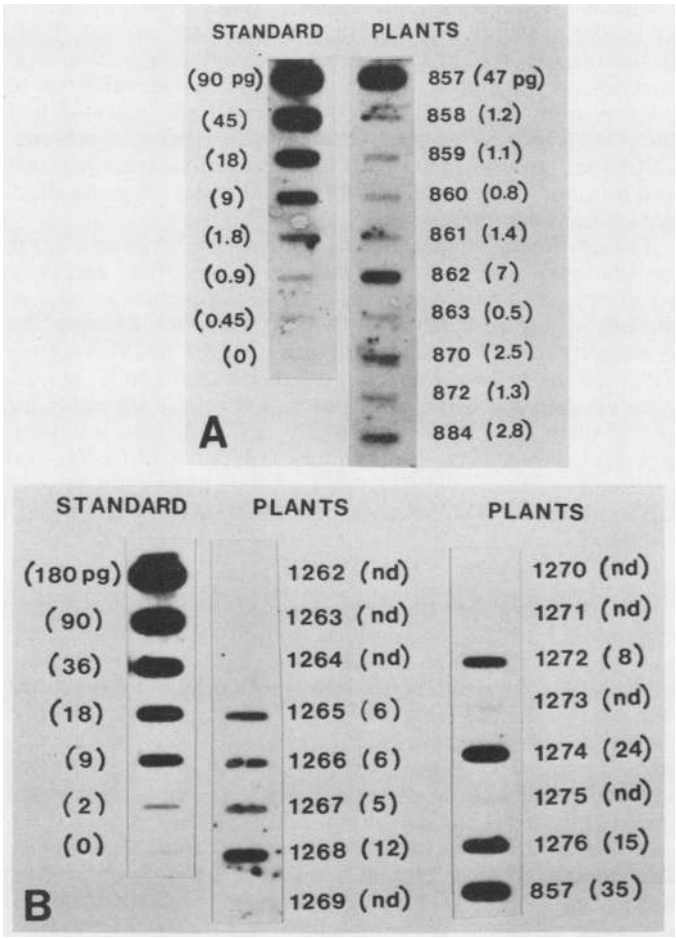

FIG. 4. RNA slot-blot analysis of regenerated plants and progeny. Total RNA (20 μg) isolated from regenerated AMVBTS plants (A) or progeny of plant No. 857 (B) were analyzed as described in "Materials and Methods." Standard toxin RNAs were synthesized as [^{3}H]RNA of defined specific activity for accurate quantification, and combined with 20 μg tobacco RNA prior to binding to filters. Labeled [^{32}P]DNA probes homologous to *B.t.* toxin were hybridized to the filters, which were visualized by autoradiography. Quantification (pg) of test plants is by densitometry on autoradiograms relative to standard transcripts; nd is "not detectable."

858 are of variable sizes, all less than single copy, and not visible in Fig. 1). Additional digests to analyze the border fragments with recombinant genes attached to plant DNA (3) indicated that each transformant with intact genes contained between one and three different inserts that hybridized at single-copy intensity (data not shown). The relative proportion of intact inserts, copy numbers, and overall frequency of regeneration in these and our other AMVBTS plants compare favorably to our experience with other genes, thereby supporting the idea that this truncated toxin does not have the deleterious effects on plant cells that we observed with the full length toxin.

Northern Blots. Northern slot-blot analyses of 10 transformants are shown in Figure 4A; hybridization intensities of sample RNAs are correlated with known quantities of synthetic *B.t.* toxin mRNAs (see "Materials and Methods"). Some of the insect-resistant plants contained less than 2 pg toxin-related RNA per 20 μg total cellular RNA, while the plant with highest expression (No. 857) showed approximately 47 pg toxin-RNA per 20 μg total RNA. This represents a 50-fold range of expression in hornworm-resistant transformants. Plants No. 858 and No. 884 showed evidence of toxin-related RNAs despite having only

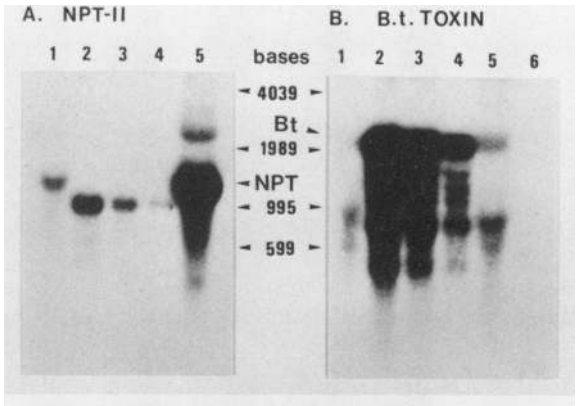

FIG. 5. Northern blot analysis of NPT-II and *B.t.* toxin gene transcripts in plant No. 1265. RNA isolated from plant No. 1265 was electrophoresed on formaldehyde formamide-containing agarose gels, transferred onto nitrocellulose and probed with [^{32}P]DNA homologous to NPT-II (A) or *B.t.* toxin (B) genes. Standard [^{3}H]RNA transcripts for NPT-II and *B.t.* toxin were used for quantification of plant RNAs; *in vitro* synthesized RNA transcripts of defined length were used as size markers (center). A, Lane 1, 20 μg total RNA of No. 1265; lanes 2 to 4, 20 μg H425 RNA plus 100, 50, and 10 pg *in vitro* synthesized NPT-II transcripts, respectively; lane 5, 17 μg oligo-dT selected RNA of No. 1265; B, lane 1, 20 μg total RNA of No. 1265; lanes 2 to 4, 20 μg H425 RNA plus 180, 90, and 35 pg *in vitro* synthesized *B.t.* toxin transcripts; lanes 5 and 6, 28 μg and 17 μg oligo-dT selected RNA of No. 1265 and H425, respectively. Locations of apparent *B.t.* toxin (Bt) and NPT-II (NPT) transcripts are noted.

broken insertions; we have not analyzed this further since only marginal insect resistance was observed in either plant (below, and Table I).

Most transformants analyzed on slot-blots for both NPT-II and toxin RNAs contained approximately 10-fold more NPT-II hybridizing RNA, on a per weight basis (data not shown). However, northern gel analysis indicated a far greater disparity between the levels of the two mRNAs of the predicted sizes, 1230 bases for NPT-II, and 2230 bases for the toxin, excluding polyadenylation (Fig. 5, A and B). While the NPT-II mRNA generally appeared to be intact and of the predicted size in all plants analyzed, the toxin RNA routinely appeared as a minor species of the predicted size, with additional significant hybridization of distinct shorter fragments. Some of the shorter fragments co-migrated with less than full-length fragments of the standard *B.t.* RNA, which was generated by *in vitro* transcription using SP6 polymerase (see "Materials and Methods"). This suggests that the toxin RNA might have one or more regions that are particularly labile. Mapping of the additional toxin-related RNAs to determine their identity is now in progress, with both *in vivo* and *in vitro* synthesized transcripts serving as substrates.

Immunoblots. Immunoblot analysis of toxin-related polypeptides in plants No. 859 and No. 860 is shown in Figure 6. A specific immunoreactive polypeptide of approximately 72,000 D co-migrates with the truncated toxin standard (*tac B.t.*). Several additional protein bands of slower mobility were also present in the plant extracts. Control plant tissues H425, not transformed with the toxin chimera, do not contain either the 72,000 D or the higher mol wt polypeptides, although other more rapidly migrating cross-reacting polypeptides were detected in varying amounts in transformants and controls. The immunoreactive polypeptides of higher mobility observed in extracts of plants No. 859 and No. 860 are likely to be aggregates of the 72,000 D toxin, since we have occasionally observed similar bands in our *E. coli*-produced toxin extracts. Based on staining intensities and comparison with *E. coli*-produced toxin, we estimate that ap-

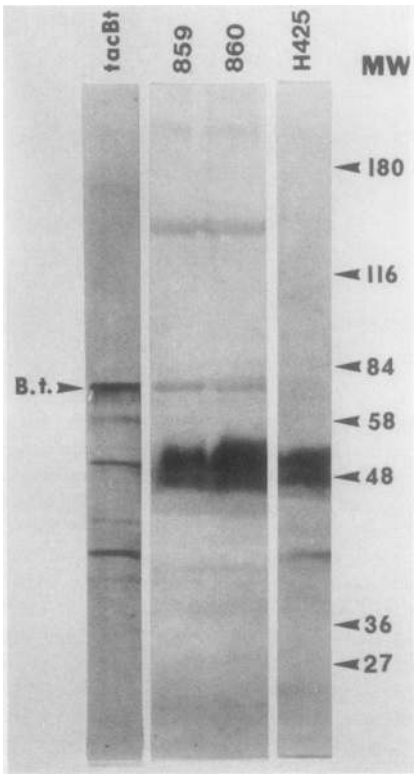

FIG. 6. Immunoblot analysis of AMVBTS plants. Total protein (80 μg) from plants No. 859, No. 860, and untransformed H425, and standard *B.t.* toxin generated in *E. coli* (*tac B.t.*) was electrophoresed on 7% polyacrylamide gels, then transferred to nitrocellulose. The blots were incubated with rabbit-anti-*B.t.* toxin serum, followed by alkaline-phosphatase-conjugated anti-rabbit immunoglobulin. Enzymic visualization of immunoreactive polypeptides was then carried out. The *E. coli*-produced truncated toxin band (*B.t.*) noted in lane 1 is estimated at approximately 5 ng, based on dye staining, immunoreactivity, and bioassays prior to dilution.

proximately 1 ng toxin protein per 80 μg total protein was present in extracts of these plants.

Analysis of Progeny. Table I presents information pertaining to larval toxicity, gene copy number, and amount of toxin mRNA in 10 regenerated transformants. A correlation between the level of toxicity in bioassays and the molecular characteristics of the toxin gene in the plants is apparent. Plants No. 858 and No. 884, both without intact toxin genes, do not show significant toxicity. The remaining eight plants were lethal to all the test larvae within the test period, and the highest toxicities were correlated with the highest levels of toxin-related transcripts.

Transmission of resistance to progeny was tested by allowing plants to flower, and seed was generated by self-pollination. Figure 3 (B and C) shows the results of Southern blot analyses of 10 seedlings derived from self-pollination of plant No. 857, which was found by additional restriction mapping to contain three independent insertions of the toxin chimera. The restriction digest generating internal toxin gene fragments (Fig. 3B) showed that nine of the progeny had one or more copies of the toxin gene. Additional restriction mapping, including border digests (Fig. 3C), revealed that various combinations of the three inserts were found in the progeny from plant No. 857. Figure 4B provides evidence that variable levels of toxin-related RNA appeared in progeny. The presence of toxin-related RNAs corresponded to the presence of one or more of the three toxin inserts in the progeny. However, the three inserts were not expressed at identical levels, since only marginal toxicity and little toxin-related RNA was seen when the insert characterized by the 1.5 kb border fragment (Fig. 4C) was the only insert present. Table II summarizes the data on insect bioassays and nucleic acid analyses for the progeny of plant No. 857. Similar analyses of progeny from additional AMVBTS plants have indicated that the AMVBTS gene routinely continues to express in progeny. Outcrosses are now in progress between various transformants and progeny to determine to what extent gene dosage regulates the level of toxin expression.

Toxicity Against Other Insects. The tobacco hornworm larvae are convenient for assays of toxicity because they are more sensitive to *B.t.* toxin than many other Lepidopteran insects, and are easy to use in a laboratory setting. However, to assess the practicality of the resistance obtained with the AMVBTS gene, we tested the resistance of toxin-producing plants to *H. zea*, *H. virescens*, and *S. exigua*. In four successive tests, using either the parent plant No. 857 or its progeny with all three insertions represented (No. 1265, for example), reductions in feeding and increased mortality of each larval type were observed relative to larvae fed control H425 tissues (data not shown).

DISCUSSION

We have generated transgenic tobacco plants that express a chimeric *B.t.* toxin gene at sufficient levels to provide resistance to Lepidopteran insects. Similar results have recently been reported (10, 27) using a crystal protein gene homologous to the one used in the present work, but differing in DNA sequence. Our results and those presented elsewhere (10, 27) indicate that the *B.t.* toxin presents unusual problems that must be overcome to obtain useful levels of toxin protein in plants. Our initial experiments led to the conclusion that expression of intact protoxin was in some way toxic to plant tissues, since transformants that contained toxin protein soon died, and no regenerated healthy transformants contained the toxin. While the truncated toxin did not show the same apparent toxicity toward plant cells, the level of toxin mRNA obtained in our insect-resistant transformants remained quite low relative to the adjacent NPT-II gene, and relative to other chimeric genes. The low level of toxin-related RNA correlates with low levels of toxin protein.

Table II. *Toxicity in Progeny of AMVBTS Plant No. 857*

Seeds resulting from self-pollination of AMVBTS plant No. 857 were germinated, and randomly selected seedlings were tested for AMVBTS insertions present (Fig. 3, B and C), toxin-related RNA content (pg/20 μg; see Fig. 4B), and for both feeding reductions relative to control tissues H425 (− to ++++) and larval mortality (no. killed/no. tested). The three possible genes in No. 857 progeny, based on border-mapping (Fig. 3C) are 'a' (3.8 kb border), 'b' (2.0 kb), and 'c' (1.5 kb).

Plant No.	Genes	RNA	Toxicity
H425	0	ND[a]	− (0/26)
1262	c	ND	− (3/6)
1263	c	ND	− (0/6)
1264	—	ND	− (0/6)
1265	a, b, c	6	++++ (6/6)
1266	a, b, c	6	++++ (6/6)
1267	a, b, c	5	++++ (6/6)
1268	a, b, c	12	++++ (6/6)
1269	c	ND	− (2/6)
1270	c	ND	− (4/6)
1271	c	ND	− (0/6)
1272	a, b, c	8	++++ (6/6)
1273	c	ND	− (2/6)
1274	a, b, c	24	++++ (6/6)
1275	c	ND	− (4/6)
1276	a, b, c	15	++++ (6/6)

[a] Not detectable.

However, due to the high potency of the *B.t.* toxin toward susceptible insects, some transformants were resistant to *M. sexta* even when toxin protein was below detectable levels.

We have introduced several unique features into the toxin gene to obtain useful levels of expression. These include a 5′ noncoding region corresponding to that of the AMV coat protein mRNA (13) and the addition of new codons at the point where the toxin coding region was artificially terminated. The utility of these features is undergoing further analysis. No accurate comparisons to alternative constructions are yet possible, since expression of chimeras prior to these modifications was too low to be detected. Our use of a plant virus noncoding region on the AMVBTS gene chimera was based on reports (11, 13) that plant viral mRNAs are exceptionally efficient in translational initiation.

Our results indicate that expression of the AMVBTS gene, based on steady state transcript levels, varies by more than 50-fold between independently derived insect-resistant transformants. Expression of the selectable marker, NPT-II, in the same transformants is consistently higher by approximately 10-fold. While the nopaline synthase promoter on the selectable marker would normally be considerably weaker than the CaMV 35S promoter (19), our positioning of the 35S enhancer between the two promoters would likely provide a bidirectional enhancement to equalize the promoter strengths. We cannot rule out the possibility that two promoters in such close proximity interact or compete for expression, but analogous constructions with reporter genes have previously shown active expression from both promoters. To compound the problem of low levels of *B.t.* toxin-related RNA the use of slot blots to quantitate toxin-specific mRNA was found to be deceptive. Northern gel hybridization analysis demonstrated that much of the toxin-related RNA was present in a low mol wt form, in RNA preparations where the NPT-II mRNA appeared quite intact. This suggests that the toxin transcript is unstable, possibly due to inefficient posttranscriptional processing or rapid turnover. We are exploring each of these possibilities by direct analysis of the toxin transcripts in plant cells and by synthetic modifications of the toxin coding sequence.

The levels of toxin present in the most actively expressing plants shown here were sufficient to demonstrate for the first time that extracts of resistant plants contain a peptide of the same size as the truncated toxin. These levels of toxin production are also sufficient to deter feeding patterns of various Lepidopteran pests, including the corn earworm, cotton bollworm, and beet armyworm. A detailed analysis of the present toxin gene expression, coupled with systematic alterations in primary and secondary characteristics of the transcript, will provide insight into problems specific to the expression of toxin protein in plants, as well as more generally to the expression of additional chimeras. Information resulting from such analysis is currently being applied to obtain cotton (26) resistant to Lepidopteran insects.

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LITERATURE CITED

1. AMANN E, JG BROSIUS 1985 ATG vectors for regulated high-level expression of cloned genes in *Escherichia coli*. Gene 40: 183–190
2. ARONSON AI, W BECKMAN, P DUNN 1986 *Bacillus thuringiensis* and related insect pathogens. Microbiol Rev 50: 1–24
3. BARTON KA, AN BINNS, AJM MATZKE, M-D CHILTON 1983 Regeneration of intact tobacco plants containing full length copies of genetically engineered T-DNA, and transmission of T-DNA to R1 progeny. Cell 32: 1033–1043
4. BIGELOW CC, M CHANNON 1982 Hydrophobicities of amino acids and proteins. *In* GD Fasman, ed, CRC Handbook of Biochemistry and Molecular Biology, Ed 3, Proteins Vol 1. CRC Press, Boca Raton, FL, pp 209–243
5. BLAKE MS, KH JOHNSTON, GJ RUSSEL-JONES, EC GOTSCHLICH 1984 A rapid sensitive method for detection of alkaline phosphatase conjugated anti-antibody on western blots. Anal Biochem 136: 175–179
6. BURNETTE WN 1981 Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein-A. Anal Biochem 112: 195–203
7. CHIRGWIN JM, AE PRZYBYLA, RJ MACDONALD, WJ RUTTER 1979 Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 24: 5294–5299
8. DELLAPORTA SL, J WOOD, JB HICKS 1985 Maize DNA miniprep. *In* R Malmberg, J Messing, I Sussex, eds, Molecular Biology of Plants: A Laboratory Course Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 36–37
9. DEPICKER A, S STACHEL, P DHAESE, P ZAMBRYSKI, HM GOODMAN 1982 Nopaline synthase: transcript mapping and DNA sequence. J Mol Appl Genet 1: 561–573
10. FISCHHOFF DA, KS BOWDISH, FJ PERLAK, PG MARRONE, SM MCCORMICK, JG NIEDERMEYER, DA DEAN, K KUSANO-KRETZMER, EJ MAYER, DE ROCHESTER, SG ROGERS, RT FRALEY 1987 Insect tolerant transgenic tomato plants. Bio/Technology 5: 807–813
11. GALLIE DR, DE SLEAT, JW WATTS, PC TURNER, T MICHAEL, A WILSON 1987 The 5′-leader sequence of tobacco mosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Res 15: 3257–3273
12. GARDNER RC, AJ HOWARTH, P HAHN, M BROWN-LUEDI, RJ SHEPHERD, J MESSING 1981 The complete nucleotide sequence of an infectious clone of cauliflower mosaic virus by M13MP7 shotgun sequencing. Nucleic Acids Res 9: 2871–2888
13. GEHRKE L, PE AURON, GJ QUIGLEY, A RICH, N SONENBERG 1983 5′-Conformation of capped alfalfa mosaic virus ribonucleic acid 4 may reflect its independence of the cap structure or of cap-binding protein for efficient translation. Biochemistry 22: 5157–5164
14. GRONENBORN B, RC GARDNER, S SCHAEFER, RJ SHEPHERD 1981 Propagation of foreign DNA in plants using cauliflower mosaic virus as vector. Nature 294: 773–776
15. HOOD EE, GL HELMER, RT FRALEY, M-D CHILTON 1986 The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. J Bacteriol 168: 1291–1301
16. JOHN ME, MC JOHN, P ASHLEY, RJ MACDONALD, ER SIMPSON, MR WATERMAN 1984 Identification and characterization of cDNA clones specific for cholesterol side-chain cleavage cytochrome P-450. Proc Natl Acad Sci USA 81: 5628–5632
17. KOZAK M 1983 Comparison of initiation of protein synthesis in procaryotes, eucaryotes, and organelles. Microbiol Rev 47: 1–45
18. MURASHIGE T, F SKOOG 1962 A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497
19. OW DW, JD JACOBS, SH HOWELL 1987 Functional regions of the cauliflower mosaic virus 35S RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. Proc Natl Acad Sci USA 84: 4870–4874
20. PERALTA EG, R HELLMISS, W REAM 1986 *Overdrive*, a T-DNA transmission enhancer on the *A. tumefaciens* tumour-inducing plasmid. EMBO J 5: 1137–1142
21. ROWE GE, A MARGARITIS 1987 Bioprocess developments in the production of bioinsecticides by *Bacillus thuringiensis*. Crit Rev Biotechnol 6: 87–127
22. SAIKI RK, S SCHARF, F FALOONA, KB MULLIS, GT HORN, HA ERLICH, N ARNHEIM 1985 Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230: 1350–1354
23. SCHNEPF HE, HR WHITELEY 1985 Delineation of a toxin-encoding segment of a *Bacillus thuringiensis* crystal protein gene. J Biol Chem 260: 6273–6280
24. SCHNEPF HE, HC WONG, HR WHITELEY 1985 The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. J Biol Chem 260: 6264–6272
25. SNEH B, S SCHUSTER, S GROSS 1983 Improvement of the insecticidal activity of *Bacillus thuringiensis* var. *entomocidus* on larvae of *Spodoptera littoralis* (Lepidoptera, Noctuidae) by addition of chitinolytic bacteria, a phagostimulant and a UV-protectant. Z Angew Entomol 96: 77–83
26. UMBECK P, G JOHNSON, K BARTON, W SWAIN 1987 Genetically transformed cotton (*Gossypium hirsutum* L.) plants. Bio/Technology 5: 263–266
27. VAECK M, A REYNAERTS, H HOFTE, S JANSENS, M DE BEUCKELEER, C DEAN, M ZABEAU, M VAN MONTAGU, J LEEMANS 1987 Transgenic plants protected from insect attack. Nature 328: 33–37
28. WHITELEY JR, HE SCHNEPF 1986 The molecular biology of parasporal crystal body formation in *Bacillus thuringiensis*. Annu Rev Microbiol 40: 549–576
29. YADAV NS, J VANDERLEYDEN, DR BENNETT, WM BARNES, M-D CHILTON 1982 Short direct repeats flank the T-DNA on a nopaline Ti plasmid. Proc Natl Acad Sci USA 79: 6322–6326