Transfer and Transcriptional Expression of Coleopteran cryIIIB Endotoxin Gene of Bacillus thuringiensis in Eggplant

Qi Chen, Gajko Jelenkovic', Chee-Kok Chin, Sharon Billings, Jodi Eherhardt, and Joseph C. Goffreda

Department of Plant Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, New Brunswick, NJ 08903

Peter Day

The Center for Molecular Biology in Agriculture, Cook College, New Jersey Agricultural Experiment Station, Rutgers University, The State University of New Jersey, New Brunswick, NJ 08903

Additional index words. Bt, Colorado potato beetle, Leptinotarsa decemlineata, gene and protein expression, insect resistance

Abstract. Three constructs of a coleopteran toxic cryIIIB Bacillus thuringiensis gene were engineered and incorporated into eggplant (Solanum melongena L.). Southern blot analysis of the eight primary transformants and segregational analysis of their R, progenies indicated that the chimeric cryIIIB constructs in each of the transgenic plants were stably incorporated at a single locus or at multiple sites within the same linkage group and that they were regularly transmissible to the progeny. The results of Northern blot and RNase protection analyses demonstrated that transcription of the cryIIIB mRNA takes place in plant cells, but only a small amount of the expected entire length transcripts were produced. The amount of the 5' end mRNA fragment produced was at least 30 to 40 times more abundant than the amount of the 3' end mRNA fragment. This could be interpreted to mean that either the two ends of the mRNA are of different stability or that the transcription process is often interrupted and only a few mRNAs complete the entire process to the end. When the transgenic plant mRNA was reverse-transcribed, amplified by polymerase chain reaction, and hybridized to the cryIIIB probe, two smaller molecular weight mRNA species were identified. Thus, the preponderance of the cryIIIB mRNA in transgenic plants exists as a truncated species, a situation similar to that of cryl genes when expressed in transgenic plants. Seedlings from the eight independent transgenic plants were tested for Coleopteran insect resistance. However, they did not demonstrate any significant resistance to the first and second instar larvae of the Colorado potato beetle (Leptinotarsa decemlineata Say).

The insecticidal crystal protein genes of Bacillus thuringiensis (Bt) have emerged as an important gene family in the biotechnological manipulation of insect resistance in cultivated plant species (McGaughey and Whalon, 1992). In their native host, these genes are expressed during bacterial sporulation, producing proteins in the form of crystals. Upon ingestion of the spores, the toxic protein crystals are solubilized and processed in the midgut of an insect larva, causing porein in the membrane of the epithelial cells and subsequently leading to mortality of the larva (English and Slatin, 1992). The crystals and spores, in various formulations and under various trade names, have been successfully used during the last 2 decades in controlling some important agricultural pests (Aronson et al., 1986). The main problem with such an approach is the high cost of the spore and crystal production and the relatively fast degradation of the toxic proteins under normal conditions of plant growth and development (Dulmage, 1980).

With the advent of a biotechnological approach to genetic plant manipulation, it has become possible to transfer and integrate Bt crystal protein genes into plant genomes and obtain a genotype resistant to a particular insect pest (Boulter et al., 1990; Brunke and Meeusen, 1991). The notable feature of these genes is that their toxic products are highly specific to a particular taxonomic group of insects. The genes are classified into four major groups according to their toxic specificity: cryI, cryII, cryIII, and cryIV, which are toxic to lepidopteran, both lepidopteran and dipteran, coleopteran, and dipteran insects, respectively (Hofte and Whiteley, 1989). A new group, cryVI, has been described recently, the products of which are active against nematodes (Feltelman et al., 1992). In addition to the specificity of their products, the genes in the same major group display considerable structural polymorphism, which is a basis for further subgrouping (Hofte and Whiteley, 1989). There are 60 Bt crystal protein genes characterized at the nucleotide level (Adang et al., 1993). CryIA(a), cryIA(b), and cryIA(c) have been introduced into tobacco (Adang et al., 1985; Barton et al., 1987; Murray et al., 1991; Perlak et al., 1990; Vaeck et al., 1987); cryIA(b) and cryIA(c) into tomato (Fischhoff et al., 1987; Perlak et al., 1990) and cotton (Perlak et al., 1990); cryIA(b) into corn (Koziel et al., 1993); cryIA(c) into potato (Cheng et al., 1992); and cryIA(b) into cranberry (Serres et al., 1992) and poplar (McCown et al., 1991). These are all genes of the lepidopteran group. When cryI genes with intact coding sequences were placed under various regulatory sequences for expression in plant cells, no detectable expression of the genes was determined (Vaeck et al., 1987). However, the chimeric constructs with truncated 3' termini of the coding region showed a low level of expression at the mRNA
and toxicity level (Barton et al., 1987; Vaeck et al., 1987). In contrast, a relatively high level of expression of the truncated cryIAb gene has been reported in transgenic tobacco grown under greenhouse and field conditions (Carozzi et al., 1992). To enhance the level of expression of cryIAb at the protein level, Perlak et al. (1991) changed the nucleotide at the third position in certain codons of the truncated coding region so that the AT to GC ratio was altered. They observed a >100-fold increase in the protein expression in transgenic tomato plants. Since the resultant amino acid sequence was almost identical to the wild type gene, it was concluded that bias in codon usage is one of the main reasons for cryI gene low expressivity in transgenic plants (Perlak et al., 1991). More drastic alterations of the codons of the same gene [cryIAb] dramatically increased its expression at the protein level in transgenic maize (Koziel et al., 1993).

At least five of the cryIII (coleopteran-specific) genes have been sequenced: cryIIIA (Hemstadt et al., 1987), cryIIIB (Donovan et al., 1988; Sick et al., 1989), cryIIIB2 (Donovan et al., 1992), cryIIIC (Peferoen et al., 1990), and cryIIID (Lambert et al., 1992). Only a few studies have been carried out on the expression of the cryIII family of genes in transgenic plants. Murray et al. (1991) reported poor transient expression of the native cryIIIA gene at the mRNA level in electroporated carrot protoplasts, and similar results were reported by Adang et al. (1985) using the same gene. When the native gene was modified for optimal expression in plant cells, the level of expression was dramatically increased and resistance of transgenic potato plants to the Colorado potato beetle (CPB) was observed (Adang et al., 1993). In a similar experiment, CryIIIA protein accumulated to 0.5% to 1.0% of the total when a synthetic cryIIIA gene was introduced into tobacco plants (Sutton et al., 1992), while only 0.001% to 0.02% CryIIIA protein was detected when the native gene was used (Barton et al., 1987; Vaeck et al., 1987).

Here we report on making cryIIIB chimeric constructs, incorporating them into eggplant genomes, and their expression at the RNA level in transgenic plants. Specifically, we were trying to incorporate the coleopteran-type cryIIIB gene and produce eggplant genotypes resistant to CPB. Such genotypes would be used for commercial planting or as breeding stock in eggplant breeding programs.

**Materials and Methods**

**Engineering the chimeric cryIIIB constructs.** A gene coding for the coleopteran insecticidal protein (cryIIIB) was made available to us by Ecogen Inc. The coding region of the insecticidal protein was delineated to the nucleotide positions 655 (5') and 2690 (3') of this gene. To express this gene in plants, the regulatory sequences controlling its expression in bacteria were deleted and replaced by those that were expected to regulate its expression in plant cells. Using the double stranded Nested Deletion Kit (Pharmacia,

---

**Fig. 1.** Structural parameters of the three cryIIIB constructs [(a) Q30; (b) Q40; (c) Q202] along with the expected fragments in southern analysis of transgenic eggplants when two probes are used (cryIIIB and uidA). Below each construct, a diagram of the expected fragment is shown depicting length and homologous sequences expected after hybridization with the probes. Homologous region plus dashed line = the expected size of a fragment. The restriction enzyme sites are as follows: E = Eco RV, Ec = Eco RI, H = Hind III, P = Pst I, Ss = Sna I, Sp = Sph I, Ss = Sst I. The prefix P = the promoter regions and the prefix T = the terminator regions.
A series of deletions at the 5' terminus were generated, differing in sequence length before the ATG start codon. These truncated forms of the cryIIIB gene were further modified by removing a 166-bp fragment by restriction with Ssp I at the 2724 nucleotide position at the 3' terminus. The truncated gene containing a 150-bp leader sequence was cloned into plasmid pCaMVCN (Pharmacia) containing the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator (NOS-T), with a deleted CAT gene. An 8-bp leader of cryIIIB was also cloned into plasmid pMJD36 containing the 35S promoter and omega translation enhancer (Gallie et al., 1987). The NOS-T was inserted as a separate fragment at the 3' end of the cryIIIB gene. Both constructs were restricted from the cloning vectors and ligated into the pBI121 plasmid (Clontech, Palo Alto, Calif.) at the unique Hind III polynucleotide site, using blunt-end ligation, between the nptII and GUS (uidA) reporter genes, in parallel and anti-parallel direction, producing four constructs (Q30, Q40, Q201, and 402). The pBI121 plasmid was then transferred into Agrobacterium tumefaciens strain LBA4404 using a direct DNA transfer system (Gynheung et al., 1988) for use in plant transformation studies. Only three (Q30, Q40, and Q202) of the four constructs were successfully used in these plant transformation studies (Fig. 1).

Transformation, regeneration, and selection. ‘Black Jack’ eggplant seeds were surface-sterilized twice: first, with 20% Clorox followed by five rinses and an overnight soak in a thin film of water, then after 24 h, with 10% Clorox followed by three rinses. This double sterilization effectively eliminated contamination caused by fungal spores that germinated in the overnight soak. Seeds were replaced in test tubes on 10 ml of half-strength Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.6% agar, and no plant growth regulators. The tubes were maintained at 26°C and a 16-h photoperiod at 50 µmol·m⁻²·s⁻¹. Seeds were germinated on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 3% sucrose, 0.6% agar, and no plant growth regulators. The tubes were maintained at 26°C and a 16-h photoperiod at 50 µmol·m⁻²·s⁻¹. When the seedlings had reached an average height of 4 cm, 15 to 18 days after germination, and before the formation of primary leaves, the seedlings were decapitated by a transverse cut through the stem below the cotyledons. The cut surface of each hypocotyl was inoculated with an Agrobacterium tumefaciens culture, either as liquid or paste. After a cocultivation time of 24 h, the antibiotic augmentin (125 µg·ml⁻¹) (Smith-Kline Beecham Pharmaceuticals, Philadelphia) was added to suppress bacterial growth. Multiple shoots were regenerated from the cut surface within 2 weeks. After about 6 weeks, when the regenerating shoots had developed two to three leaves, tissue samples from an apical leaf were harvested and tested for the presence of β-glucuronidase (GUS) activity (Jefferson, 1987). The shoots were excised and placed in MS medium containing Augmentin (300 µg·ml⁻¹) for rooting. Once rooted, the plants were transferred to the greenhouse. We described details of eggplant transformation procedures elsewhere (unpublished data).

Seeds obtained by selfing the primary transformants (R₁) were germinated and the progeny produced (R₂) were tested for expression of the GUS reporter gene.

**Southern blot analysis.** Genomic DNA of eggplant was isolated from young leaf tissue using the Junghans and Matzlaff (1990) procedure. Five to ten micrograms of DNA was restricted with either Eco RV or Hind III and run on a 0.9% agarose gel. The DNA was blotted onto a nylon (Nytran) membrane according to Maniatis (1989). Two different DNA probes were used in filter hybridization: a) a 1.2-Kb cryIIIB fragment from the 5' end to the Hind III site; b) the entire 1.8-Kb coding sequence of the GUS reporter gene restricted from pBI211 (Clontech) with Sst I and Xba I. The probes were labeled with the oligo labeling kit (Pharmacia) and hybridization was done using 2 × 10⁶ cpm/ml of the P³²-dCTP probe. Hybridization, washing, and autoradiogram development was done as described by Maniatis (1989).

**Northern blot analysis.** Total RNA was isolated from young leaves of eggplant using the method of DeVries et al. (1988). Poly (A)⁺ mRNA was separated from total RNA with an oligo (dt) cellulose column according to a method described by Kingston (1989). From 3 to 19 µg of mRNA was electrophoresed on a denaturing gel and transferred to a nylon membrane (Gene Screen; DuPont, Boston). Hybridization was carried out according to methods described by Selden (1989).

**RNase protection assays.** RNA probes for RNase protection assays were transcribed and labeled in vitro with ³²P-CTP using a kit (Riboprobe Gemini II; Promega, Madison, Wis.). Two DNA fragments were used as templates for this in vitro transcription. They were obtained in the following way. Plasmid pGE34, a recombinant of pGEM 32 and the cryIIIB gene, was restricted with Eco RV and Pvu II, which produced a fragment containing 376 bp of the most distal portion of the cryIIIB gene, a deleted CAT gene. An 8-bp leader of cryIIIB, with the coding region, was cloned into plasmid pBI121 containing the cauliflower mosaic virus (CaMV) 35S promoter and nopaline synthase terminator (NOS-T), with a deleted CAT gene. The NOS-T was inserted as a separate fragment at the 3' end of the cryIIIB gene. Both constructs were restricted from the cloning vectors and ligated into the pBI121 plasmid (Clontech, Palo Alto, Calif.) at the unique Hind III polynucleotide site, using blunt-end ligation, between the nptII and GUS (uidA) reporter genes, in parallel and anti-parallel direction, producing four constructs (Q30, Q40, Q201, and 402). The pBI121 plasmid was then transferred into Agrobacterium tumefaciens strain LBA4404 using a direct DNA transfer system (Gynheung et al., 1988) for use in plant transformation studies. Only three (Q30, Q40, and Q202) of the four constructs were successfully used in these plant transformation studies (Fig. 1).

In vitro reverse transcription and cDNA amplification with polymerase chain reaction (PCR). One microgram of mRNA from transgenic eggplant specimen 430-2 and 1 µg from a control were reverse-transcribed into the first cDNA strand using a 3' system kit (RACE; GIBCO-BRL, Gaithersburg, Md.) and further amplified by Taq DNA polymerase using PCR. The PCR amplification conditions were 94°C for 1 min and 45°C for 2 min for 35 cycles. An oligo dT (17-mer) and a 26-mer oligonucleotide derived from the cryIIIB gene were further amplified using 2 × 10⁶ cpm/ml of the P³²-dCTP probe. Hybridization, washing, and autoradiogram development was done as described above.

**Fig. 2.** Plasmid pGE34, containing the cryIIIB gene, from which 5' and 3' riboprobes were derived for RNase protection assays. Striped and shaded areas represents the 5' and 3' ends of cryIIIB, respectively, and were used as riboprobes in RNase protection assays.
Insect toxicity assays. Leaf petioles of the primary transgenic eggplants were inserted into wet oasis medium to preserve leaf turgidity, then placed in petri dishes. Two first- or second-instar larvae were placed in each petri dish on the leaves. The amount of leaf tissue consumed by the larvae and the rate of insect development were observed and compared with insects on control (‘Black Jack’) leaves. Five leaves from each genotype were tested per experiment and the experiments were repeated three times. In addition, control seedlings (‘Black Jack’) and about 400 R, seedlings, including GUS-positive and GUS-negative segregants from all original transformants, were individually caged and inoculated with first instar larvae in the growth chamber when the plants had six primary leaves. They were rated for resistance as described below.

Results

GUS assays of eggplant transformants and their derivatives. Eight transgenic eggplants produced with the seedling decapitation method were identified with the GUS assay, representing three of the constructs. This transformation method was used because of the high rate of shoot regeneration (100%) in initial experiments. However, due to an extremely low frequency of transformants (<0.1%), this approach has been replaced by using leaf explant tissue with selectable medium (unpublished data). The transformed plants were successively numbered according to the original A. tumefaciens strains used (Fig. 1). The transformants appeared normal in phenotype and growth habit, and they reached the reproductive phase showing no visible abnormality in fruit and seed set in the greenhouse.

Stability and transmissibility of the transferred constructs. The pattern of segregation for GUS activity in most of the R, progenies indicate that, in each independent transformant, the integration of the cryIIIB constructs appears to be confined to a single locus in the genome (Table 1). Segregation in Q40-1 progen is different in that most R, seedlings were of a negative phenotype (Table 1). Segregants of Q30-1 demonstrated a preponderance of GUS-negative plants. We cannot offer a definite interpretation of this data at this time.

Southern blot analysis. Southern blot analysis was used to ascertain the physical integration of the cryIIIB constructs and their organization in the chromosomes of the transgenic genotypes. When plant DNA was restricted with Eco RV, the cryIIIB probe was expected to hybridize to the fragment between the Eco RV sites in the truncated cryIIIB gene and its 35S promoter and to produce a single band of about 2.0 Kb on the autoradiograph. Indeed, this band was observed in all GUS-positive genotypes (Figs. 1 and 3a). Two additional bands (not shown) were observed for all figures is 1) control, nontransgenic sample, 2) 30-1, 3) 30-2, 4) 30-3, 5) 30-4, 6) 30-5, 7) 30-6, 8) 40-1, 9) 202-1.

Since there is only one Hind III restriction site in the constructs, located in the middle of the cryIIIB gene, digestion of the DNA from transformed eggplant genotypes with Hind III would produce two large fragments, carrying the left and right borders of T-DNA (defined as the left and right fragments, respectively), the size depending on the positions of the nearest Hind III sites in the transgenic plant genome. Since the cryIIIB probe consisted of the 5' Hind III segment of the construct, hybridization with the plant DNA should produce one band with one insertion and more than one band, if more insertions are present. Therefore, the Hind III bands would differ in number, size, and intensity, depending on the number and place of insertion (Fig. 1). Four of the six analyzed genotypes of the Q30 series displayed a single band between 7 and 10 Kb.
10 Kb. The genotype Q30-3 displayed six bands indicating multiple cryIIIB inserts. A similar interpretation is offered for Q30-1, which displayed two bands (Fig. 3b). The GUS probe was expected to hybridize to the left fragment of the Hind III restricted DNA of the transgenic plants. In the case of Q40 and Q202, the produced bands should be the same size as the ones produced with the cryIIIB probe (Figs. 1 b and c and 3c). Conversely, with the Q30 series, the two bands were of different sizes (Figs. 1a and 3c).

RNA expression of the coleopteran cryIIIB gene. In Northern blot assays, the genotypes Q30-1, Q30-2, Q30-4, and Q40-1 displayed weak signals at 1.2 to 1.4 Kb (Fig. 4a), while the remaining transgenic plants, along with the control, showed no hybridization signals. In the RNase protection experiments, hybridization with an antisense RNA fragment at the 5' terminus produced a major band of about 150 bp in all tested genotypes except for the control (Fig. 4b). The intensity of the displayed signals was noticeably higher in four of the genotypes, with the weakest from Q30-3. In contrast, hybridization with an antisense RNA fragment at the 3' terminus revealed signals in only five of the tested genotypes; these signals were conspicuously lower in intensity compared to those from the 5' terminus (Fig. 4c). The position of this band corresponds to the expected fragment of about 300 bp.

The cDNA amplified from the mRNA reverse transcripts of the transgenic 430-2 also hybridized-with the cryIIIB gene fragment probe that was used in our Southern and Northern hybridization experiments. Two distinct bands, corresponding to fragments of about 1.2 Kb and 0.6 Kb respectively, were present on the autoradiograph, whereas the control mRNA-amplified DNA did not show any bands. These results demonstrate that transcription of cryIIIB mRNA takes place in the transgenic eggplants. However, a band of the expected transcript size of 1.8 Kb is not observed on this autoradiograph; rather, two smaller molecular species whose origin is unknown at this time were observed (Fig. 5). Further studies on the stability of the cryIIIB mRNA are needed to understand the origin of the smaller molecular species in the transgenic plants.

Insect bioassay. Insect bioassays on R, leaves in petri dishes or whole R, plants under growth-chamber conditions demonstrated no noticeable difference in the quantity of consumed leaf areas between various transgenic and control eggplants. Likewise, the feeding pattern of the larvae on transgenic plants appeared to be the same as on control plants. Once initiated by a larva at a given spot, feeding continued at the same place, producing a large hole in the leaf, sometimes encompassing 30% of the entire leaf area. Four to five days after the insect inoculations, the leaves of plants were severely damaged in both types of assays, and the whole plants were almost completely defoliated after 8 to 10 days. The larvae continued their feeding on the main stem of the plants. Parallel with such voracious consumptions of leaf tissue, the larvae seemed to proceed with their normal growth and development, with no observable differences in the mortality between those feeding on the leaves of transgenic plants and those feeding on the control plants. These observations indicate, rather convincingly, that the consumption of the leaf tissue of transgenic eggplant is not inhibitory to the growth and development of CPB larvae.

Discussion

The evidence obtained by Southern analysis of the transgenic eggplants indicate that each of the tested genotypes is distinguishable by a unique banding profile on the autoradiograms, confirming their derivation from independent transformation events. Furthermore, this analysis shows that most (five) of the transgenic eggplants most likely contain a single copy of the cryIIIB construct. This agrees with previous reports showing that single-copy inserts are prevalent among transgenic genotypes (Budar et al.,
result from multiple insertions at different loci along the chromo-
926 J. A
somes or at the same locus. The origin of multiplicity at a single
bling of the involved constructs or that they are organized in a
restricted fragments and their variability in signal intensity. This
would suggest that there has been some rearrangement or scram-
bbling of the involved constructs or that they are organized in a
cluster with chromosomal DNA dispersed between the constructs.
Segregational analysis of the R, progenies, showing a Mendelian pattern for the GUS (uidA) gene, provides further evidence that
integration of the cryIIIB constructs in all transgenic eggplants
(except Q40-1) is confined to a single locus or to multiple sites
within the same linkage group. Also, it has been demonstrated that
the chimeric constructs are stably incorporated into eggplant genomes and are transmissible to progeny in a regular manner.

Our analysis of the steady-state level of the coleopteran cryIIIB
mRNA in the transgenic eggplants failed to reveal the expected
molecular weight mRNA by the Northern blot procedure. Instead of a 2-Kb band, a smaller sized band (about 1.2 Kb) was observed
for four of the seven tested transgenic genotypes. In most past
studies of this kind (Barton et al., 1987; Vaeck et al., 1987), the
presence of mRNA of expected molecular weight has been de-
tected, although truncated mRNAs of lower molecular weight seem to have been the most abundant form of lepidopteran cryl
mRNA. It appears that some of the intact cryIIIB mRNA may be
present in our transgenic materials in amounts below the level of
detection by Northern analysis. The signal detection of the 3’
cryIIIB mRNA by our RNase protection assay strongly supports
such an assertion. Thus, the expression of the coleopteran cryIIIB
gene at the mRNA level seems to mirror the expression of the
lepidopteran-type cryl gene. This is in spite of the fact that the
toxin-coding region of the coleopteran cryIIIB gene shares only
about 50% sequence homology to the lepidopteran cryl genes
(Hofte and Whiteley, 1989).

The origin of truncated cry mRNA in transgenic plants, al-
though a very common occurrence, is poorly understood. Murray
et al. (1991) carried out a thorough study of the kinetics of
transcription of the lepidopteran genes in electroporated carrot
protoplasts and-in transgenic tobacco and determined that tran-
cipated species are the result of mRNA instability. They suggested that
degradation of 5’→3’ or 3’→5’ mRNA by exonuclease activity
may lead to short transcripts observed on the Northern blots.
Furthermore, among the various 3’ end cry deletions they tested,
the highest accumulation of cryl transcript was observed with the
longest segment, i.e., a construct containing 579 nucleotides of the
5’ portion of the gene. Results of our RNase protection assay are
consistent with their finding that the transcript of the 5’ portion
of the gene is most abundant in crytransgenic plants; in our case, there
is perhaps a 15- to 20-fold difference in the amount of mRNA
between the distal portions of 5’ and 3’ of the coding region. This
pattern of appearance of transcriptional signals could be explained
by postulating that 3’ exonuclease activity is considerably higher
than 5’e exonuclease activity in degradation of the mRNA. Alterna-
tively, the 5’ distal transcripts could be the result of premature
transcription termination at either a specific site or various sites,
and the 3’ transcripts are the consequence of an occasional full-
length transcription of the entire coding region. The use of a
cascade of riboprobes in RNase protection assays may reveal the
problems associated with cry mRNA transcription and stability. It
appears then, that great similarities exist in the expression of
lepidopteran and coleopteran cry genes at the mRNA level in
transgenic plants. However, a difference between these two groups
of genes emerged in biotoxicity tests. Most of the feeding experi-
ments using transgenic material containing the lepidopteran cryl
gene detected toxicity to a variety of lepidopteran insects, in partic-
ular, to Mundaclia sexta (Adang et al., 1985; Barton et al.,
1987; Cheng et al., 1992). In contrast, we have failed to observe
any crippling effect on CPB with transgenic material containing the
Coleopteran cryIIIB gene. This discrepancy probably is due to the
sensitivity of various insects to the toxic protein in the plants
(Donovan, personal communication). However, lower concentra-
tions of toxic protein in the leaf tissue cannot be ruled out.

Recent studies suggest that expression of cry genes at the
protein level is greatly enhanced by using a synthetic gene, with
altered nucleotides at the third positions of the codons, and
possessing other characteristics of eucaryotic genes (Adang et al.,
1993; Sutton et al., 1992). Presently, we are pursuing eggplant
transformation studies with a synthetic cryIIIA gene.

Literature Cited

Adang, M.J., E. Firoozabady, J. Klein, D. DeBoer, V. Sekar, J. D. Kemp,
E. Murray, T. A. Rocheleau, K. Rashka, G. Stafford, C. Stock, D. Sutton,
and D. J. Merlo. 1985. Expression of a Bacillus thuringiensis insecticidal
protein gene in tobacco plants. p. 345-353. In: C. J. Arntzen and C. Ryan (eds). Molecular strategies for crop protection. Alan R.
Liss, New York.

Adang, M.J., M.S. Brody, G. Cardinale, N. Eagan, R.T. Rouch, C.K.
Shevemaker, A. Jones, J.V. Oakes, and K.E. McBride. 1993. The
reconstruction and expression of a Bacillus thuringiensis crylA gene in
protoplasts and potato plants. Plant Mol. Biol. 21: 1131–1145.

Aronson, AL, W. Beckman, and P. Dunn. 1986. Bacillus thuringiensis
and related insect pathogens. Microbiol. Rev. 50: 1-24.

Barton, K.A., H.R. Whiteley, and N.S. Yang. 1987. Bacillus thuringiensis
s-endotoxin expressed in transgenic Nicotiana tabacum provides resis-
tance to Lepidopteran insects. Plant Physiol. 85: 1103-1 109.

Boulter, D., J.A. Gatehouse, A.M.R. Gatehouse, and V.A. Hilder. 1990.
Genetic engineering of plants for insect resistance. Endeavor 14:185-
190.

Brunke, K.J. and R.L. Meuesen. 1991. Insect control with genetically
engineered crops. Trends Biotechnol. 9:197-200.

Budar, F., L. Thia-Toong, M. VanMontagu, and J.P. Hernalsteens. 1986.
Agrobacterium-mediated gene transfer results mainly in transgenic
plants transmitting T-DNA as a single mendelian factor. Genetics
114:303-313.

Carozzi, N.B., G.W. Warren, N. Desai, S.M. Jayne, R. Lotstein, D.A.
Rice, E. Evola, and M.G. Koziel. 1992. Expression of a chimeric CaMV
35S Bacillus thuringiensis insecticidal protein gene in transgenic to-
bacco. Plant Mol. Biol. 20:539-548.

Cheng, J., M.G. Bolyard, R.C. Saxena RC, and M.B Sticklen. 1992.
Production of insect resistant potato by genetic transformation with a
delta endotoxin gene from Bacillus thuringiensis var. kurstaki. Plant Sci.
81:83-91.

Deroles, S.L., and R.C. Gardner. 1988a. Expression and inheritance of
kanamycin resistance in a large number of transgenic petunias generated
by Agrobacterium-mediated transformation. Plant Mol. Biol. 11:355-
364.

Deroles, S.C. and R.C. Gardner. 1988b. Analysis of the T-DNA structure
in a large number of transgenic petunias generated by Agrobacterium-
mediated transformation. Plant Mol. Biol. 11:365-377.

DeVries, S., H. Hoge, and T. Bisseling. 1988. Isolation of total and
polysomal RNA from plant tissues, p. B6:1-13. In: S. Gelvin and R.
Schilperoort (eds.). Plant molecular biology manual.

Donovan, W.P., J.M. Gonzalez, M.P. Gilbert, and C. Dankocsik C. 1988.
Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. Mol. Gen. Genet. 214:365-372.

Donovan, W.P., M.J. Rupar, A.C. Slaney, T. Malvar, C. Gawron-Burke, and T. Johnson. 1992. Characterization of two genes encoding *Bacillus thuringiensis* insecticidal crystal proteins toxic to coleoptera species. Appl. Environ. Microbiol. 58:3921-3927.

Dulmage, H.T. 1980. Insecticidal activity of isolates of *Bacillus thuringiensis* and their potential for pest control. In: H.D. Burges (ed.). Microbial control of pest and plant diseases 1970-1980. Academic Press, London. p. 193-222.

English, L. and S. Slatin. 1992. Mode of action of delta-endotoxins from *Bacillus thuringiensis*: a comparison with other bacterial toxins. Insect Biochem. Mol. Biol. 22:1-7.

Feitelson, J.S., J. Payne, and L. Kim. 1992. *Bacillus thuringiensis*: insects and beyond. Bio/Technology 10:271-275.

Fischhoff, D.A., K.S. Bowdish, F.J. Perlak, P.J. Marrone, S.M. McCormick, J.G. Niedermeyer, D.A. Dean, K.K. Kusano-Kretzmer, E.J. Mayer, D.E. Rochester, S.G. Rogers, and R.T. Fraley. 1987. Insect tolerant transgenic tomato plants. Bio/Technology 5:807-813.

Gallie, D.R., D.E. Sleat, J.W. Watts, P.C. Turner, and M.A. Wilson. 1987. The S- leader sequence of tobaccomosaic virus RNA enhances the expression of foreign gene transcripts *in vitro* and *in vivo*. Nucleic Acids Res. 15:3257-3273.

Gynheung, A., P.R. Ebert, A. Mitra, and S.B. Ha. 1988. Binary vectors. In: J.R. Funk and D. Farkas (eds.). Plant molecular biology manual. Kluwer, Belgium. p. A37.

Heberle-Bors, E., B. Charvat, D. Thompson, J.P. Schernthaner, A. Barta, A.J.M. Matzke, and M.A. Matzke. 1988. Genetic analysis of T-DNA insertions into the tobacco genome. Plant Cell Rpt. 7:571-574.

Hernstadt, C., T.E. Gilroy, L.A. Sobieski, B.D. Bennett, and D.H. Gaertner. 1987. Nucleotide sequence and deduced amino acid sequence of a coleopteran-active delta endotoxin from *Bacillus thuringiensis*. Subsp. *C58* derivatives. Mol. Gen. Genet. 207:471-485.

Jorgensen, R., C. Snyder, and J.D.G. Jones. 1987. T-DNA is organized predominately in inverted repeat structures in plants transformed with *Agrobacterium tumefaciens* C58 derivatives. Mol. Gen. Genet. 207:471-477.

Junghans, H. and M. Metzlaff. 1990. A simple method for the preparation of total plant DNA. BioTechniques 8:176.

Kingston, R.E. 1989. Preparation of poly (A)+ RNA. p. 4.5.1-4.5.2. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.). Current protocols in molecular biology. Wiley, New York.

Koziel, M.G., G.L. Beland, C. Bowman, N.B. Carozzi, R. Crenshaw, L. Crossland, J. Dawson, N. Desai, M. Hill, S. Kadwell, K. Launis, K. Lewis, D. Maddox, K. McPherson, M.R. Meghjii, E. Merlin, R. Rhodes, G.W. Warren, M. Wright, and S.V. Evola. 1993. Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Bio/Technology 11:194-200.

Lambert, B., V. Theunis, R. Agud, K. Van Audenhove, C. Decock, S. Janssen, J. Seuvink, and M. Peferoen. 1992. Nucleotide sequence of gene *cryIIID* encoding a novel coleopteran-active crystal protein from strain Bt 109p of *Bacillus thuringiensis* Subsp. *curtisi* strain Bt 109p. Genbank accession number X8170.

Maniatis, T., E.F. Fritsch, and J. Sambrook. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press.

McCown, H.B., D.E. McCabe, D.R. Russell, D.J. Robinson, K.A. Barton, and K.F. Raffa. 1991. Stable transformation of *Populus* and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rpt. 9:590-594.

McGaughey, W.H. and M.E. Whalon. 1992. Managing insect resistance to *Bacillus thuringiensis* toxins. Science 258:1451-1455.

Murai, S. and F. Skoog. 1982. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 55:473-497.

Murray, E.E., T. Rocheleau, M. Eberle, C. Stock, V. Sekar, and M. Adang. 1991. Analysis of unstable RNA transcripts of insecticidal crystal protein genes of *Bacillus thuringiensis* in transgenic plants and electroporated protoplasts. Plant Mol. Biol. 16:1035-1050.

Peferoen, M., B. Lambert, and H. Joos. 1990. New strains of *Bacillus thuringiensis*. Eur. Pat. Publ. no. 382990.

Perlak, F.J., R.W. Deaton, T.A. Armstrong, R.L. Fuchs, S.R. Sims, J.T. Greenplate, and D.A. Fischhoff. 1990. Insect resistant cotton plants. Bio/Technology 8:939-943.

Perlak, F.J., R.L. Fuchs, D.A. Dean, S.L. McPherson, and D.A. Fischhoff. 1991. Modification of the coding sequence enhances plant expression of insect control protein genes. Proc. Natl. Acad. Sci. USA 88:3324-3328.

Rogers, S.G. and R.T. Fraley. 1987. Insect tolerant transgenic tomato plants. J. Amer. Soc. Hort. Sci. 120(6):921-927. 1995.

Sick, A., R. Gaertner, and A. Wong. 1989. Nucleotide sequence of a Coleoptera-active toxin gene from a new isolate of *Bacillus thuringiensis* subsp. tolworthi. Nucleic Acids Res. 18:1305.

Sutton, D.W., P.K. Havstad, and J.D. Kemp. 1992. Synthetic *cryIIA* gene from *Bacillus thuringiensis* improved for high expression in plants. Transgenic Res. 1:228-236.

Vaek, M., A. Reynaerts, H. Hofte, S. Janssen, M. DeBeuckeleer, C. Decock, S. Janssen, J. Seuvink, and M. Peferoen. 1992. Nucleotide sequence of gene *cryIIID* encoding a novel coleopteran-active crystal protein from strain Bt 109p of *Bacillus thuringiensis* Subsp. *curtisi* strain Bt 109p. Genbank accession number X8170.