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Madhuri Kota

Henry Daniell
University of Pennsylvania

Sam Varma

Stephen F. Garczynski

Fred Gould

See next page for additional authors

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Abstract

Evolving levels of resistance in insects to the bioinsecticide *Bacillus thuringiensis* (Bt) can be dramatically reduced through the genetic engineering of chloroplasts in plants. When transgenic tobacco leaves expressing Cry2Aa2 protoxin in chloroplasts were fed to susceptible, Cry1A-resistant (20,000- to 40,000-fold) and Cry2Aa2-resistant (330- to 393-fold) tobacco budworm *Heliothis virescens*, cotton bollworm *Helicoverpa zea*, and the beet armyworm *Spodoptera exigua*, 100% mortality was observed against all insect species and strains. Cry2Aa2 was chosen for this study because of its toxicity to many economically important insect pests, relatively low levels of cross-resistance against Cry1A-resistant insects, and its expression as a protoxin instead of a toxin because of its relatively small size (65 kDa). Southern blot analysis confirmed stable integration of *cry*2Aa2 into all of the chloroplast genomes (5,000–10,000 copies per cell) of transgenic plants. Transformed tobacco leaves expressed Cry2Aa2 protoxin at levels between 2% and 3% of total soluble protein, 20- to 30-fold higher levels than current commercial nuclear transgenic plants. These results suggest that plants expressing high levels of a nonhomologous Bt protein should be able to overcome or at the very least, significantly delay, broad spectrum Bt-resistance development in the field.

Keywords
chloroplast transformation, insecticide resistance, resistance management

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Author(s)

Madhuri Kota, Henry Daniell, Sam Varma, Stephen F. Garczynski, Fred Gould, and William J. Moar

Overexpression of the *Bacillus thuringiensis* (Bt) Cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects

(chloroplast transformation/insecticide resistance/resistance management)

Madhuri Kota*, Henry Daniell†‡, Sam Varma†, Stephen F. Garczynski*, Fred Gould§, and William J. Moar*¶

Departments of *Entomology and †Molecular Genetics Program, Botany and Microbiology, Auburn University, Auburn, AL 36849; and §Department of Entomology, North Carolina State University, Raleigh, NC 27695

ABSTRACT Evolving levels of resistance in insects to the bioinsecticide *Bacillus thuringiensis* (Bt) can be dramatically reduced through the genetic engineering of chloroplasts in plants. When transgenic tobacco leaves expressing Cry2Aa2 protoxin in chloroplasts were fed to susceptible, Cry1A-resistant (20,000- to 40,000-fold) and Cry2Aa2-resistant (330- to 393-fold) tobacco budworm *Heliothis virescens*, cotton bollworm *Helicoverpa zea*, and the beet armyworm *Spodoptera exigua*, 100% mortality was observed against all insect species and strains. Cry2Aa2 was chosen for this study because of its toxicity to many economically important insect pests, relatively low levels of cross-resistance against Cry1A-resistant insects, and its expression as a protoxin instead of a toxin because of its relatively small size (65 kDa). Southern blot analysis confirmed stable integration of *cry*2Aa2 into all of the chloroplast genomes (5,000–10,000 copies per cell) of transgenic plants. Transformed tobacco leaves expressed Cry2Aa2 protoxin at levels between 2% and 3% of total soluble protein, 20- to 30-fold higher levels than current commercial nuclear transgenic plants. These results suggest that plants expressing high levels of a nonhomologous Bt protein should be able to overcome or at the very least, significantly delay, broad spectrum Bt-resistance development in the field.

The use of commercial, nuclear transgenic crops expressing *Bacillus thuringiensis* (Bt) toxins has escalated in recent years because of their advantages over traditional chemical insecticides. However, in crops with several target pests with varying degrees of susceptibility to Bt (e.g., cotton), there is concern regarding the suboptimal production of toxin, resulting in reduced efficacy and increased risk of Bt resistance (1, 2). Additionally, reliance on a single (or similar) Bt protein(s) for insect control increases the likelihood of Bt-resistance development (3). Plant-specific recommendations to reduce Bt-resistance development include increasing Bt expression levels (high-dose strategy), expressing multiple toxins (gene pyramiding), or expressing the protein only in tissues highly sensitive to damage (tissue-specific expression) (4).

Expression of economically important genes via the chloroplast has been reported for insect (Bt Cry1Ac) and herbicide (glyphosate) resistance with much higher expression levels than nuclear transgenic plants (5, 6). For a recent historical overview of chloroplast transformation see Daniell *et al.* (6). Besides extremely high protein levels, chloroplast gene expression also results in tissue specificity occurring predominantly where functional plastids are present. Chloroplast transformation uses two flanking sequences that, through homologous recombination, insert foreign DNA into the spacer region between the functional genes of the chloroplast genome, thus targeting the foreign genes to a precise location. Such precise targeting eliminates the "position effect" frequently observed in nuclear transgenic plants. The maternal inheritance of the chloroplast genome in most crops also reduces the potential for outcrossing of foreign genes to other plants (especially weedy species) (6).

Most current commercial transgenic plants in the United States that target lepidopteran pests contain either Cry1Ab (corn) or Cry1Ac (cotton) (7, 8). Bt corn is targeted primarily against European corn borer *Ostrinia nubilalis* (Hübner), although other pests such as the corn earworm *Helicoverpa zea* (Boddie) also may be affected. Bt cotton is targeted primarily against the tobacco budworm *Heliothis virescens* (F.), pink bollworm *Pectinophora gossypiella* (Saunders), and *H. zea*. Especially with cotton, other pests such as *Spodoptera* spp. also can be economically damaging, but have only limited susceptibility to Cry1Ac. Use of single Bt proteins to control insects such as *H. virescens* and *H. zea* could lead to relatively rapid Bt-resistance development (4, 9). Additionally, because Cry1Ab and Cry1Ac share more than 90% protein homology (10) resistance to one Cry1A protein most likely would confer resistance to another Cry1A protein as seen in *H. virescens* (9, 11). Nowhere is this threat of resistance more of a concern than with *H. zea,* which usually feeds on corn in the spring and early summer, then migrates over to cotton to complete several more generations (4). Clearly, different Bt proteins are needed to decrease the development of resistance.

Another class of Bt proteins that are toxic to many lepidoptera such as *O. nubilalis* and *H. virescens* but exhibit limited homology to Cry1A proteins are the Cry2A proteins (10, 12–15). The atomic structure, ion channel formation, and binding of Cry2A is different from Cry1 toxins (16), and Cry2A is already under commercial development (nuclear transformation) (17). Although, one strain of Cry1Ac-resistant *H. virescens* (Cp73) exhibited a high level of cross-resistance to Cry2A (9), another strain of Cry1Ac-resistant *H. virescens* (YHD2) exhibited only slight cross-resistance to Cry2A, and it was suggested that the major genetic and biochemical mechanisms responsible for resistance to Cry1Ac in these two strains are likely different (11).

We report here the overexpression of the Cry2Aa2 (18) protoxin in tobacco chloroplasts and a possible solution to the evolution of resistance to Bt observed in the field. Cry2Aa2

Abbreviations: Bt, *Bacillus thuringiensis*; RR, resistant ratio; FL, fiducial limits.
‡Present address: Department of Molecular Biology and Microbiology, BIO 306, University of Central Florida, Orlando, FL 32816-2360.
¶To whom reprint requests should be addressed at: Department of Entomology, 301 Funchess Hall, Auburn University, Auburn, AL 36849. e-mail: wmoar@acesag.auburn.edu.

was chosen because of its high toxicity against many insect pests and small size of the protoxin (65 kDa) compared with the Cry1A protoxins (130–135 kDa). Although Bt protoxins are assumed to be more environmentally stable than toxins, most current transgenic corn and cotton contain essentially the Bt toxin because gene size can be a limiting factor for optimal expression in plants (7, 8). Further, through the use of protoxin, the collateral damage to nontarget insects is minimized (19).

MATERIALS AND METHODS

Construction of a Chloroplast Expression Vector and Plant Transformation. The tobacco chloroplast expression vector pZS-KM-cry2A was constructed by inserting a 2.2-kb *Sma*I/*Hinc*II fragment from pMTS-6 containing *cry*2A2 into a Klenow-filled and dephosphorylated *Spe*I-digested pZS-197 (15, 20). This plasmid was amplified in *Escherichia coli* XL1-Blue (Stratagene). Generation of chloroplast transgenic tobacco (*Nicotiana tabacum* var. Petit Havana) was carried out as described (6, 21). Green calli and shoot formation were observed after about 5–8 weeks of selection. Leaf segments were allowed to grow and produce additional leaflets under continued selection for another 4–5 weeks. About six shoots that were still in physical contact with the selection medium were selected and transferred to bottles or test tubes with rooting medium (21). Only those shoots with intact root primordia developed roots under selection. Shoots with sufficient leaves and roots were transferred to autoclaved potted soil and grown in growth chambers.

Primer Construction and PCR Analysis. To distinguish Cry2Aa2-chloroplast transgenics from mutants, PCR was performed on total DNA extracted from transformed plants. PCR primers were designed by using OMEGA (IntelliGenetics). Primer oligomers corresponding to *rbc*L5′ (5′-CAAGTGTT-GGATTCAAAGCTGGTGT-3′), *rbc*L3′ (5′-GGACATCCT-TGGGGTATGCGC-3′), and *aad*A (5′-AATGGTGACTTC-TACAGCGCGGAGA-3′) were obtained from Genosys (The Woodlands, TX). Total leaf DNA from unbombarded and putative transgenic plants was isolated (22). PCRs were performed as suggested by the manufacturer by using a GeneAmp PCR system 2400 (Perkin–Elmer). Samples were carried through 30 cycles by using the following temperature sequence: 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min. Cycles were preceded by denaturation for 5 min at 94°C and a final extension cycle at 72°C for 7 min. PCR products were electrophoresed through a 0.7% agarose gel.

Southern Blot Analysis. Integration of foreign genes into the chloroplast genome and cell copy number was determined by Southern blot analysis. Total plant DNA from untransformed and transgenic plants was isolated by using the cetyltrimethylammonium bromide procedure (23). Ten micrograms of DNA per sample was digested with *Eco*RV and separated on a 0.7% agarose gel, then transferred to a nylon membrane. This process was repeated, generating a total of six blots. Two different probes were used, one to determine foreign gene integration (chloroplast border sequences) and the other to estimate gene copy number (*cry*2Aa2). Hybridization was performed by using 18×10^6 mCurie ^{32}P-labeled DNA (24).

Immunoblot Analysis. To confirm *cry*2Aa2 expression in leaf tissue, Western blot analysis was done. Proteins were extracted from 100 mg of leaf material (excluding veins and leaf midrib). Fresh tissue was homogenized in extraction buffer (50 mM Tris·HCl, pH 8.0/2% SDS/2 mM β-mercaptoethanol/0.1 mg/ml phenylmethylsulfonyl fluoride). To determine expression levels, solubilized Cry2Aa2 (0.0002–2.0 μg), expressed and purified from *E. coli*, was used for comparisons (15). Protein concentrations were determined by using the Bradford assay with BSA as the protein standard (Bio-Rad) (25). Ten micrograms of total soluble protein was loaded per lane and electrophoresed in a 10% SDS/PAGE gel. Proteins were transferred to a nylon membrane and incubated with Cry2A polyclonal antibodies (1:25,000 dilution) (26). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad) was used (1:10,000 dilution). The Cry2Aa2 concentration from leaf material was determined by comparing the intensity of 65-kDa bands by using QUANTISCAN gel scanning software (Biosoft, Milltown, NJ).

Insect Bioassays. Bioassays were conducted either against resistant and susceptible *H. virescens* or against *H. zea* and *Spodoptera exigua* (Hübner). All *H. virescens* were obtained from the laboratory of F.G. and *H. zea* and *S. exigua* from the laboratory of W.J.M. *H. virescens* colonies were: YDK (susceptible); YHD₂ 1000MVP, a *H. virescens* strain (YHD2) that has been further selected for resistance to Cry1Ac (20,000- to 40,000-fold) (ref. 11; F.G., unpublished data); and CxC 1000IIA, a Cry1Ac-resistant *H. virescens* strain that was selected for Cry2Aa2 cross-resistance and exhibits up to a 393-fold resistance to Cry2Aa2 (ref. 9; data presented below). The CxC 1000IIA line was derived from the CP73 strain (9). The CP73 strain was crossed to a susceptible strain (CPN) and then selected on artificial diet containing Cry2Aa2 protoxin. At the time of experiments reported here the CxC strain had been selected on Cry2Aa2 for more than 24 generations. The current selection regime involved placing neonate larvae on diets containing 200 or 1,000 μg/ml of Cry2Aa2 (15). Larvae surviving after 5–7 days always were transferred to nontoxic diets for further rearing. Bioassays conducted with Cry2Aa2 in August 1998 in F.G.'s laboratory showed a resistant ratio (RR) of 393 between YDK and CxC 1000IIA [LC$_{50}$ (μg/ml) for YDK = 2.15 (95%FL = 1.23–3.06) slope = 3.30; LC$_{50}$ (μg/ml) for CxC 1000IIA = 843 (95%FL = 535-1960) slope = 1.87]. Similar bioassays with Cry1Ac gave a RR of 104 between YDK and C X C 1000IIA [LC$_{50}$ (μg/ml) for YDK = 0.13 (95%FL = 0.08–0.22) slope = 1.76; LC$_{50}$ (μg/ml) for CxC 1000IIA = 13.4 (95%FL = 7.38–23.06) slope = 1.14], where FL is fiducial limits.

H. virescens were subjected to artificial diet-incorporation bioassays (27) concurrently with leaf-disc bioassays to confirm susceptibility and resistance. Cry1Ac and Cry2Aa2 were produced as single proteins in *E. coli* (28). Protein concentrations used against *H. virescens* were: YDK, Cry1Ac (1 μg/g) and Cry2Aa2 (1.0, 2.0, 2.5, 5.0, 10.0, and 25.0 μg/g); YHD₂ 1000MVP, Cry1Ac (1.0, 5.0, 10.0, 25.0, and 50.0 μg/g), and Cry2Aa2 (1.0, 2.0, 3.5, 5.0, 10.0, 25.0, 50.0, 100.0, 200.0, and 400.0 μg/g); CxC 1000IIA, Cry1Ac (1.0, 5.0, 10.0, 25.0, and 50.0 μg/g), and Cry2Aa2 (25.0, 50.0, 100.0, 250.0, 500.0, and 1,000.0 μg/g). Mortality was assessed after 5 days. Bioassays were conducted at least twice for all treatments. Data were analyzed by using POLO (29). RR's were generated by dividing the LC$_{50}$'s of the resistant colony by the LC$_{50}$ of YDK. When the highest concentration tested resulted in less than 50% mortality, the highest concentration tested was then used as the LC$_{50}$ for generating RR.

Leaf bioassays were conducted on about 2-cm² excised leaf material and placed on distilled water-soaked cardboard lids in 50 × 12-mm plastic Petri dishes with tight-fitting lids. Five to 10 neonates were assayed per sample, with two samples per treatment, and evaluated daily for mortality for 5 days. Treatments were replicated at least twice, but 4–5 times in most cases. Preliminary leaf-disc bioassays also were performed by using transformed leaf tissue containing the *aad*A gene product, but without Cry2Aa2, to confirm that no insecticidal activity could be attributed to the transformed plant in the absence of Cry2Aa2.

RESULTS

Construction of a Chloroplast Expression Vector and Plant Transformation. The plasmid pZS-197 contains the chimeric

aminoglycoside 3′-adenyltransferase (*aad*A) gene that confers resistance to spectinomycin-streptomycin and integrates gene(s) of interest into the large single copy region of the tobacco chloroplast genome at the intergenic spacer region between *rbc*L and *acc*D (20). Integration occurs through two homologous recombination events between the chloroplast border sequences and the corresponding homologous sequences of the chloroplast genome (20). The *aad*A and *cry*2Aa2 genes in pZS-KM-cry2A are driven by the chloroplast promoter *Prr*n, and transcription is terminated by *psb*A3′ untranslated region. *Prr*n is a constitutive promoter of the rRNA operon, and *psb*A3′ untranslated region functions as a strong terminator. Because of similar protein synthetic machinery between chloroplasts and *E. coli* (30), *cry*2Aa2 expression in pZS-KM-cry2A was analyzed by Western blots using Cry2A antibodies. This analysis showed the presence of the 65-kDa Cry2Aa2 protein (data not shown). Tobacco leaves then were bombarded with pZS-KM-cry2A DNA-coated tungsten particles and placed on RMOP medium (21), with no antibiotic selection pressure, for 2 days. This procedure was to allow for aminoglycoside 3′-adenyltransferase accumulation in tissues so as to exhibit phenotypic resistance when placed on RMOP medium containing spectinomycin dihydrochloride (500 mg/liter), after day 2. The 5-mm^2 bombarded leaf pieces placed on the selection medium enlarged in size and were bleached of all color. Green calli (spectinomycin resistant) emerged from underneath (bombarded surface) the bleached calli. Green calli later grew into green shoots with leaflets. Thirteen putative transformants were obtained from 13 bombarded leaves in experiment 1 and three from experiment 2.

Foreign Gene Integration. The strategy used to determine integration of the foreign gene specifically into the chloroplast genome was two-tiered. Initially putative transgenics were screened by using PCR. Primers were designed to confirm incorporation of the gene cassette into the chloroplast genome. The primer on the plus strand, designated *rbc*L 5′, landed on the chloroplast genome upstream of the chloroplast border used for homologous recombination (refer to Fig. 1*A*). The *rbc*L5′ box represents the primer sequence, and the perpendicular dotted line is the region on the native chloroplast genome where this primer lands. The minus strand primer lands on *aad*A. In Fig. 1*A* landing of this primer is represented by the *aad*A box (the perpendicular dotted line represents the region on *aad*A where the primer lands). The rationale for this strategy is that there can be no 2.1-kb PCR product unless there is site-specific integration between *rbc*L and *acc*D (Fig. 1*A*, the thick dotted lines show the site of integration in the tobacco chloroplast genome). Therefore, the presence of a 2.1-kb PCR product in three of the 16 putative transgenics confirmed the site-specific integration of the heterologous gene cassette. To investigate the possibility of random priming, the homology of *rbc*L5′ and *aad*A primers to all known gene sequences was checked in all existing databases; no homology was observed for the *aad*A primer, and homology to the *rbc*L primers was observed only in the chloroplast genome. Therefore, no random PCR product was predicted by this analysis, as confirmed by the lack of PCR products in the untransformed plants.

The three positive clones were further analyzed with Southern hybridization to further confirm the site-specific integration of *cry*2Aa2 and to establish copy number. In the chloroplast genome, *Eco*RV sites flank the chloroplast border sequence 5′ of *rbc*L and 3′ of *acc*D (Fig. 1*A*), which generates a 3-kb fragment when digested with *Eco*RV. A transformed chloroplast genome has the foreign gene cassette inserted between *rbc*L and *acc*D, which increases the size of the *Eco*RV-digested fragment to 6 kb (Fig. 1, the thick dotted lines represent the site of integration).

Total DNA from each clone and untransformed tobacco were digested with *Eco*RV, and two blots were made from
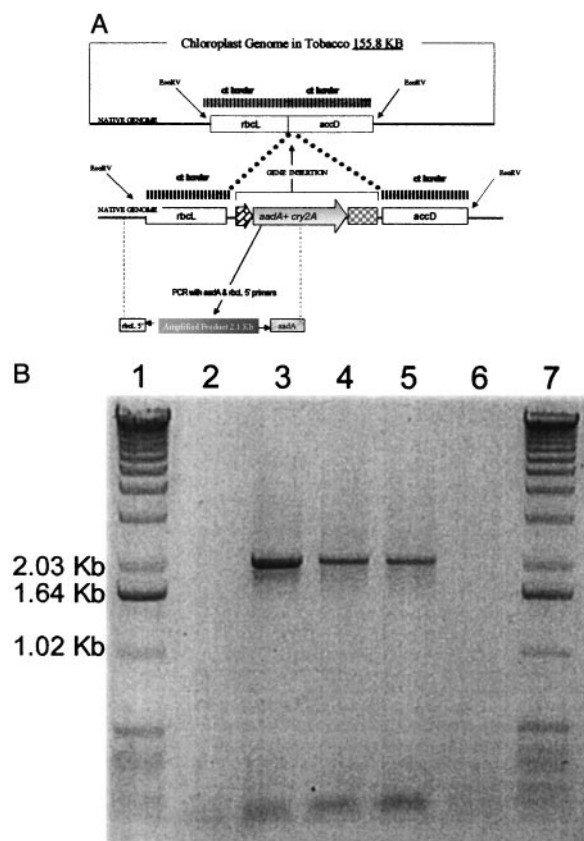

FIG. 1.   (*A*) Structure of the chloroplast genome with the site of foreign integration represented by a shaded dotted line. (*B*) PCR analysis to screen for chloroplast transformants. Primers used: *rbc*L 5′ region and *aad*A. Lanes 1 and 8, 1-kb ladder; template DNA from lane 2, nontransformed tobacco, lanes 3–6, clones 2, 5, 7 and 8, respectively; and lane 7, plasmid pZS-KM-cry2A.

these samples (Fig. 2). The blot shown in Fig. 2*A* was probed with a ^{32}P random primer-labeled 2.9-kb *rbc*L-*aac*D DNA fragment. The probe hybridized with the control as expected, lighting up a 3-kb fragment, characteristic of all untransformed chloroplast genomes. For clones 2 and 5, only a 6-kb fragment was generated, showing that all plastid genomes had the gene cassette inserted between *rbc*L and *acc*D. The number of chloroplast genomes per cell is between 5,000 and 10,000. By establishing that digestion of the chloroplast genomes of clones 2 and 5 generated only a 6-kb fragment, we confirmed homoplasmy (identical plastid genomes) and simultaneously determined the *cry*2Aa2 copy number to be between 5,000 and 10,000. This observation explains the high levels of protoxin observed in our transgenic tobacco plants. In clone 7 the probe hybridized with a 3-kb and an anomalous smaller fragment, indicating heteroplasmy and rearrangement, resulting in partial deletion of the expression cassette. The blot shown in Fig. 2*B* was probed with a ^{32}P random primer-labeled *Bgl*II–*Hin*dIII gene fragment from *cry*2Aa2. The control plant showed no hybridizing fragments (*cry*2Aa2 not present in tobacco), nor did clone 7 (confirming deletion). Clones 2 and 5 showed presence of the gene. This observation was supported by subsequent leaf bioassays where clones 2 and 5, but not clone 7, were toxic to *H. virescens*.

Protein Expression. To confirm and quantify *cry*2Aa2 expression, total soluble protein obtained from transformed and nontransformed leaves was subjected to Western blot analysis. From the results obtained by using titration curves of *E. coli*-expressed Cry2Aa2 and gel scanning, Cry2Aa2 is expressed between 2% and 3% of total soluble leaf protein (Fig. 3).
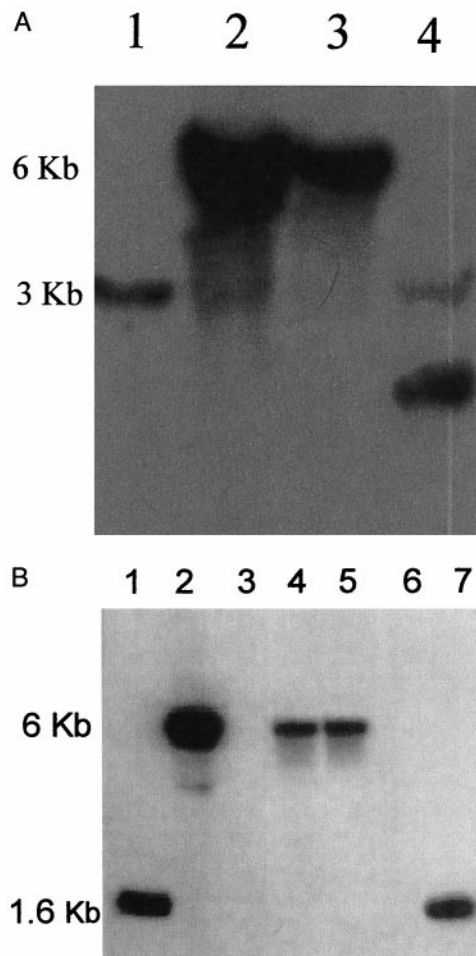
Fig. 2. (*A*) Southern blot analysis to confirm homoplasmy. Hybridization of *Eco*RV-digested total genomic DNA, with *Eco*RV/*Sst*I-digested chloroplast border flanking sequences. DNA from lane 1, nontransformed tobacco and lanes 2–4, DNA from clones 2, 5, and 7, respectively. (*B*) Southern blot analysis to confirm chloroplast integration of *cry*2Aa2. Hybridization of *Eco*RV-digested total genomic DNA, with *Bgl*II/*Hin*dIII-digested *cry*2Aa2 probe. Lanes 1 and 7, 1-kb ladder; DNA from lane 2, plasmid pZS-KM-cry2A; lane 3, nontransformed tobacco, and lanes 4–6, clones 2, 5, and 7, respectively.

Insect Bioassays. To test the toxicity of transformed tissue to insects, preliminary leaf-disc bioassays were conducted with leaflets collected from first- and second-generation clones. Clones 2 and 5, but not clone 7, were toxic to lepidopteran larvae, (in agreement with Southern blot analysis, data not shown). Additionally, leaves containing pZS-197, (pZS-KM-cry2A minus *cry*2Aa2) were nontoxic to lepidoptera.

Diet-incorporation bioassays were conducted concurrently to confirm the susceptibility reported for the various insect species and strains and to ensure that Bt-resistant genotypes also were phenotypically resistant to Bt. Because insects and Bt proteins were limited in availability, methodologies for some bioassays were reduced. Bioassays conducted against *H. virescens* confirmed that YDK was susceptible to Cry1Ac and Cry2Aa2 at concentrations similar to those previously reported [100% mortality observed by using Cry1Ac (1 μg/ml); Cry2Aa2 LC_{50} (μg/ml) = 3.03 (95%FL = 0.8–5.41) slope = 1.42 (SEM = 0.235) n = 240]; YHD2 1000MVP was highly resistant to Cry1Ac and marginally resistant to Cry2Aa2 (0% mortality observed at 50 μg/ml Cry1Ac; Cry2Aa2 LC_{50} (μg/ml) = 138 (95%FL = N/A) slope = 1.64 (SEM = 0.251) n = 241]; and CxC 1000IIA was highly resistant to both Cry1Ac and Cry2Aa2 [0% mortality observed at 50 μg/ml Cry1Ac; 0% mortality observed at 1,000 μg/ml Cry2Aa2) (Tables 1 and

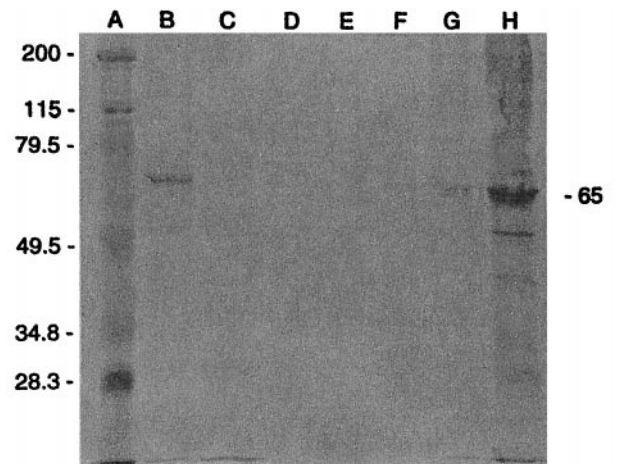Fig. 3. Immunoblot analysis. Lane A, molecular weight marker; lanes B and C, 10 μg of total soluble leaf proteins from clone 2 and nontransformed tobacco, respectively; lanes D-H, dilutions (0.0002, 0.002, 0.02, 0.2, and 2.0 μg, respectively) of solubilized Cry2Aa2 protoxin.

2). Based on these results, RR's were generated for both YHD2 and C X C 1000IIA (Tables 1 and 2).

Plant Tests. *H. virescens.* There was 100% mortality of neonate YDK feeding on clone 2 leaves, and the leaf pieces were essentially intact, whereas the control leaf pieces were completely devoured (Fig. 4). Similar results were obtained with YHD₂ 1000MVP, and CxC 1000IIA (Fig. 4.).

Bioassays also were conducted by using YDK and CxC 1000IIA that were reared on control leaves or artificial diet for 5 days (about 2nd–3rd instar), and then moved to transgenic leaves. Even these older larvae that are more tolerant than neonates (31) showed 100% mortality (data not shown).

Other insects tested. When leaves from clone 2 were fed to *H. zea* and *S. exigua*, 100% mortality was observed, whereas there was no mortality observed in the control, and the entire leaf piece was eaten (Fig. 5). Although there was no detectable feeding damage on clone 2 with *H. zea*, there was some leaf damage observed with *S. exigua*, indicative of the high tolerance of this insect species to Cry2Aa2 (15, 17).

DISCUSSION

This study reports high expression levels of Cry2Aa2 protoxin in tobacco chloroplasts. Several reasons should have contributed for such high levels of expression in comparison to those observed by using nuclear expression. Chloroplasts are prokaryotic compartments inside eukaryotic plant cells, the prokaryotic codon composition of *cry*2Aa2, high copy number of *cry*2Aa2 genes per cell, and the small size of the protoxin gene (24). This level could be further increased with the doubling of gene dosage by inserting *cry*2Aa2 into the inverted repeat region of the chloroplast genome instead of the single copy region. It is not clear whether the high *cry*2Aa2 expression levels observed in chloroplasts resulted in the formation of

Table 1. Toxicity of Cry1Ac protoxins against *H. virescens* using artificial diet incorporation bioassays

Insect, *H. virescens*	Cry1Ac LC_{50}, μg/ml		Resistance ratio	
	Reported*	Observed	Reported*	Observed
YDK	0.13	<1†	N/A	N/A
YHD₂ 1000MVP	20,000–40,000	>50	20,000–40,000	>100
CxC 1000IIA	13.4	>50	104	>100

N/A, not available.
*See text for references.
†100% mortality observed at 1 μg/ml.

Table 2.   Toxicity of Cry2Aa2 protoxins against *H. virescens* using artificial diet incorporation bioassays

Insect, *H. virescens*	Toxin	Cry2ALC$_{50}$, µg/ml		Resistance ratio	
		Reported*	Observed	Reported*	Observed
YDK	Solubilized	N/A	14.5	N/A	N/A
YDK	Inclusion bodies	0.5–5	3.0	N/A	N/A
YHD$_2$ 1000MVP	Solubilized	N/A	138.0	3–25	9.5
CxC 1000IIA	Inclusion bodies	843	0% mortality at 1,000 µg/ml	200–393	>330

N/A, not available.
*See text for references.

crystals. Studies in *E. coli* suggest that ORFs upstream of *cry*2Aa2 (within the *cry*2Aa2 operon) are required for folding Cry2Aa2 proteins to form crystals (32, 33). If crystals are desired for enhanced stability, the entire *cry*2Aa2 operon should be expressed in chloroplasts, because chloroplasts routinely express and process polycistrons (33). Yet another advantage of expressing insecticidal proteins in chloroplasts is tissue specificity. Most caterpillars feed on green tissues that are rich in chloroplasts, thereby consuming the highest level of insecticidal protein. Several chloroplast genes are light regulated and hence chloroplasts express high levels of proteins compared with plastids in nongreen tissues.

With the successful introduction of *cry*2Aa2 into the chloroplast genome, the high-dose strategy should be attainable for insects such as *H. virescens*, *H. zea,* and *S. exigua*. This study shows 100% mortality of both Bt-susceptible and Cry1Ac-resistant and Cry2Aa2-resistant *H. virescens*. We show that neonate insects, highly resistant to Bt, were killed by using Bt-transgenic leaf material even though *H. virescens* is less sensitive to Cry2Aa2 than Cry1Ac (1, 15). These results also are promising when related to reports showing marginal to high levels of cross-resistance to Cry2Aa2 (9, 11). This study also shows 100% mortality of neonate *H. zea* that contrasts with Bt cotton (Cry1Ac) efficacy against *H. zea*. The inefficient control of *H. zea* also might result in faster development of Bt resistance because a moderate level of suppression (25–50% mortality) can increase the probability of resistance development (4, 34). In this context, plants expressing *cry*2Aa2 through the chloroplast either singly, or as part of a gene-pyramid with other insect proteins (preferably non-Bt proteins with different modes of action), could become an invaluable tool for resistance management.

Bilang and Potrykus (35) recently have discussed several requirements for transforming chloroplasts of useful crops (35). One of the major limitations is the lack of knowledge of chloroplast genome sequences to locate spacer regions and transcriptional units to target site-specific integration of foreign genes. For example, it is important to transform cotton chloroplasts with *cry*2Aa2 for insect control. However, not much is known about the cotton chloroplast genome. To overcome this limitation, Daniell *et al.* (6) recently have developed a universal vector that can transform any chloroplast genome because it integrates into a highly conserved region.

Another limitation is the ability to regenerate plants only from embryonic tissues in cereals and not from mesophyll cells. Cells from embryonic tissues contain only proplastids and not
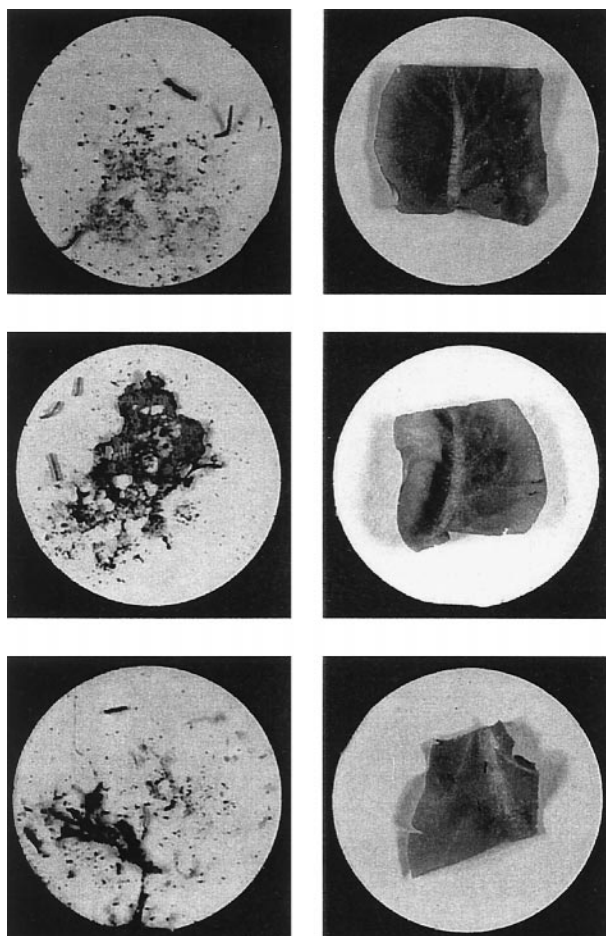

FIG. 4.   Leaf bioassay of control (*Left*) and Cry2Aa2 chloroplast transgenic tobacco leaves (*Right*) assayed against various *H. virescens* strains. YDK, susceptible (*Top*), YHD$_2$ 1000MVP, Cry1Ac-resistant (*Middle*), and CxC 1000IIA, Cry2Aa2-resistant (*Bottom*). Photographs were taken on day 4 of the assay.
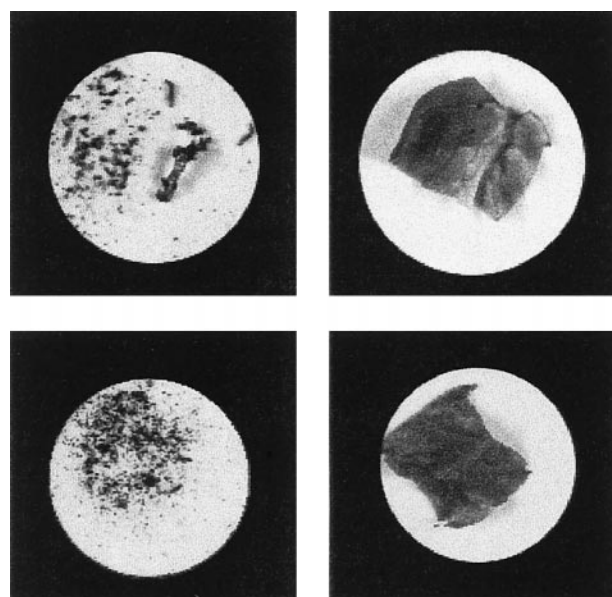

FIG. 5.   Leaf bioassay of control (*Left*) and Cry2Aa2 chloroplast transgenic tobacco leaves (*Right*) assayed against *H. zea* (*Upper*) and *S. exigua* (*Lower*). Photographs were taken on day 4 of the assay.

mature plastids. It has been suggested that these plastids are smaller than the size of microprojectiles used for DNA delivery and therefore may pose problems in transformation experiments. Successful expression of chloramphenicol acetyl transferase in proplastids of NT1 cells (36) and β-glucuronidase in proplastids of wheat embryos (37) via particle bombardment suggest that particle size may not be a problem in transforming proplastids. Some of the challenges in transforming agronomically useful crops include optimization of tissue culture techniques and the selection process to obtain transgenic plants via particle bombardment, especially from nongreen tissues. Even if homoplasmy is not obtained in the first generation, it could be accomplished in subsequent generations by germination of F_1 seeds under appropriate selection. Furthermore, high levels of expression of *cry*2Aa2 in transgenic tobacco have not affected growth rates, photosynthesis, chlorophyll content, flowering, or seed setting as observed in the laboratory. However, long-term tests using agronomically important crops grown under field conditions are needed before validation of this potential new methodology can be obtained.

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