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Publisher's version / Version de l'éditeur:
https://doi.org/10.1007/s11248-004-7714-3
Transgenic Research, 14, 3, pp. 289-298, 2005

Transgenic Research (2005) 14:289–298
DOI 10.1007/s11248-004-7714-3

The Bt gene *cry2Aa2* driven by a tissue specific ST-LS1 promoter from potato effectively controls *Heliothis virescens*

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Received 15 September 2004; accepted 15 December 2004

Key words: Bacillus thuringiensis, Cry2Aa2, Green tissue specific, *Heliothis virescens*, *Maruca vitrata*, ST-LS1

Abstract

Expression of the Cry2Aa2 protein was targeted specifically to the green tissues of transgenic tobacco *Nicotiana tabacum* cv. Xanthi plants. This deployment was achieved by using the promoter region of the gene encoding the *Solanum tuberosum* leaf and stem specific (ST-LS1) protein. The accumulated levels of toxin in the leaves were found to be effective in achieving 100% mortality of *Heliothis virescens* larvae. The levels of Cry2Aa2 expression in the leaves of these transgenic plants were up to 0.21% of the total soluble proteins. Bioassays with R_1 transgenic plants indicated the inheritance of *cry2Aa2* in the progeny plants. Tissue-specific expression of the Bt toxin in transgenic plants may help in controlling the potential occurrence of insect resistance by limiting the amount of toxin to only predated tissues. The results reported here validate the use of the ST-LS1 gene promoter for a targeted expression of Bt toxins in green tissues of plants.

Introduction

Several genes derived from *Bacillus thuringiensis*, Bt, have been successfully incorporated into the genomes of commercial crops such as cotton, maize, potato and rice (Shu et al., 2000; Nester et al., 2002). Expression of the Bt toxins confers upon these crops insect resistance without the requirement of traditional chemical insecticides. Crops carrying Bt-toxin genes are grown predominantly in Argentina, Australia, Canada, China, Mexico, South Africa and the USA (Toenniessen et al., 2003; High et al., 2004). Bt crop hectarage has been increasing rapidly with simultaneous incorporation of new crops under the Bt 'umbrella' (James, 2003). This rapid increase is, however, alarming with a potential build up of resistance in the target insects against their respective toxin.

Among many approaches employed in the successful deployment of Bt transgenic plants the use of an appropriate promoter is crucial to reducing the amount of entomocides in the field biomass. Several promoters have been used according to the deployment strategy of the transgenic crops and the target insects. They comprise promoters

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for constitutive as well as spatial and temporal expression of proteins. The 35S promoter from Cauliflower Mosaic Virus and the maize ubiquitin promoter represent a group of promoters that have a proven value for constitutive expression of Cry proteins (Ramesh et al., 2004). A second group of promoters has been successfully utilized to regulate expression of Cry proteins in plants: *phosphoenolpyruvate carboxylase* (PEPC) gene promoter, maize pollen specific promoter (Datta et al., 1998) and *calcium dependent protein kinase* (CDPK) gene promoter (Alinia et al., 2000). The intrinsic specificity of the latter group of promoters limits their application to monocots and phloem. The *ribulose-bisphosphate carboxylase* small subunit gene promoter (*rbcS*) has been shown to drive green tissue specific expression of Bt protein in tobacco but at low levels (Christov et al., 1999). The same promoter from *Chrysanthemum*, on the other hand, has been reported as a strong promoter for a hybrid Bt gene in potato (Naimov et al., 2003). A detailed study of this promoter describing the relative expression of Bt in other plant parts is however still awaited.

The leaf tissue specific promoter of a gene encoding a light inducible protein ST-LS1 from potato has been reported to express heterologous genes in the green tissues of plants (Stockhaus et al., 1987; van Voorthuysen et al., 2000). They, however, studied metabolic enzymes not required at a high dose. It would be worth investigating whether this promoter could also drive light-regulated expression of a Bt protein in plants at sufficiently high levels for effective control of target insects. In the present study, we have evaluated the expression of the Cry2Aa2 protein under the control of the ST-LS1 promoter in the green tissues of transgenic tobacco plants.

Materials and methods

Construction of the cry2Aa2 cassette for expression in tobacco

The promoter region of ST-LS1 gene from potato and the *nos* terminator sequence were generous gifts from Menachem Keller, Vitality Biotechnologies Inc, Israel. The ST-LS1 gene promoter was originally reported by Stockhaus et al. (1987, 1989). In potato, the endogenous ST-LS1 gene encodes a 10 kDa protein in the oxygen-evolving complex of photosystem II. The ST-LS1 gene promoter and the nos terminator were subcloned into the plasmid pRD400 (Datla et al., 1992) using *Eco*RI and *Hin*dIII restriction sites. The resulting plasmid was named pSTnos. The plant codon-optimized *cry2Aa2* coding sequence, contained in the pMP153 plasmid, was synthesized at the Biotechnology Research Institute, NRC, Canada. The protein sequence for the synthetic gene was based on the amino acid sequence of the Cry2Aa2 from Bt subsp. *kurstaki* (strain HD-1) (Crickmore et al., 1998; Maqbool et al., 1998). The plasmid pSTnos was digested with *Bam*HI and the cohesive ends of the DNA fragment were filled in using the DNA polymerase I (Klenow). Digestion of pMP153 with *Xba*I and *Bam*HI released the *cry2Aa2* coding sequence and its cohesive ends were filled in using the DNA polymerase I (Klenow). The blunt end fragment of *cry2Aa2* was subcloned downstream of the ST-LS1 promoter in pSTnos. The expression cassette contained the ST-LS1 promoter, the *cry2Aa2* coding sequence and the *nos* terminator sequence. The construct was named p2AST (Figure 1). DNA manipulations were performed according to standard protocols (Sambrook and Russell, 2001).

Transformation of tobacco plants

The plasmid p2AST was used to transform the *Agrobacterium* strain LBA4404 using a standard protocol (Horsch et al., 1985). These *Agrobacterium* cells were used to transform *Nicotiana tabacum* cultivar Xanthi. The transformation and regeneration procedures were those as previously published (Horsch et al., 1985; Albani et al., 1992). The regenerated plants were subsequently transferred to the greenhouse and maintained through seed set for further analysis.

Polymerase chain reaction

The transgenic tobacco plants were screened for the presence of *cry2Aa2* by PCR analysis. Genomic DNA from kanamycin-resistant plants was isolated using the Plant DNeasy mini kit from Qiagen. The primers used to detect the *cry2Aa2* gene were: forward primer (P1) 5′-AGAGGCGGC TATGACTGG-3′ and reverse primer (P2) 5′-ATCGCCATGGGACGAGAT-3′. These

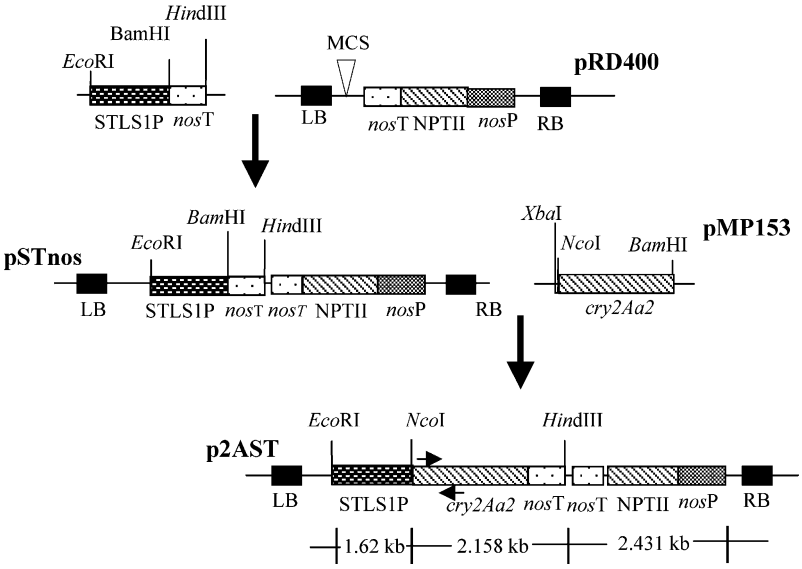

Figure 1. The schematic construction of p2AST. The DNA fragment of ST-LS1 promoter (1.62 kb) with *nos* (256 bp) terminator was subcloned into the plasmid pRD400 using *Eco*RI and *Hin*dIII restriction sites in MCS. The resulting plasmid was named pSTnos. The plasmid pSTnos was digested with *Bam*HI and the cohesive ends of the DNA fragment were filled in using the DNA polymerase I (Klenow). Digestion of pMP153 with *Xba*I and *Bam*HI released the *cry2Aa2* coding sequence (1902 bp) and its cohesive ends were filled in using the DNA polymerase I (Klenow). The blunt end fragment of *cry2Aa2* was subcloned downstream of the ST-LS1 promoter in pSTnos. The expression cassette contained the ST-LS1 promoter, the *cry2Aa2* coding sequence and the *nos* terminator sequence. The construct was named p2AST. The arrows shown in p2AST correspond to the primers used to amplify a 0.8 kb DNA fragment. The amplified product labeled with DIG was used in Southern blot analyses. MCS, Multiple Cloning Sites; LB and RB, the Left and the Right Borders of T-DNA region, respectively; *nos*T, *nos* terminator; NPTII, neomycin phosphotransferase II coding sequence; *nos*P, *nos* promoter; STLS1P, ST-LS1 gene promoter.

primers amplify an 800 bp fragment from the *cry2Aa2* gene (Figure 1). Total plant DNA extracted from fresh tobacco leaves was used as a template and the following reaction mixture was prepared for this experiment. The PCR reactions were performed in a total volume of 25 µl, comprising 50 ng of tobacco genomic DNA, 25 pmol of each primer, 250 µM of each dNTP, 0.5 unit Taq DNA polymerase, 1.5 mM $MgCl_2$ and 2.5 µl 10X PCR buffer. DNA was denatured at 94°C for 5 min followed by 35 amplification cycles (94°C for 45 s, 62°C for 45 s, 72°C for 45 s).

Southern analysis

To determine the number of independent loci of transgene and integration in tobacco plants Southern blot analyses were performed. Genomic DNA was isolated from young leaf tissues using a Plant DNeasy kit (Qiagen). The genomic DNA was restricted with *Hin*dIII or *Eco*RI, which cut the T-DNA at unique sites (Figure 1). Standard procedures were followed for Southern blot analysis (Sambrook & Russell, 2001). Purified DNA (40 µg) was digested with *Hin*dIII or *Eco*RI, separated by gel electrophoresis on a 0.8% TAE-agarose gel and capillary blotted onto nylon membrane (Amersham). The probe for Southern hybridization was prepared by labeling a 0.8 kb *cry2Aa2* fragment with digoxygenin (Roche) by PCR. The primers used in the amplification step were those mentioned in the previous section. Conditions for hybridization, and washing were those recommended by the manufacturer. The bands were detected immunologically using the anti-DIG-alkaline phosphatase conjugate and the colorimetric substrate NBT/BCIP.

Protein expression analyses

Protein was extracted from fresh leaves using a method described by Stewart et al. (1996) with a slight modification. Fresh leaf tissue was ground in liquid nitrogen and 0.2 g was resuspended in 400 µl of 0.1 N NaOH. The sample was incubated for 30 min on ice, 80 µl of 1 M Tris–HC1

(pH 4.5) was added for neutralization. Each sample was clarified by centrifugation at 14,000 × *g* at 22°C. To degrade or remove the non-Cry protein background, trypsin (5% of total protein) was added to the solubilized protein extract in the supernatant and incubated at 37°C for 30 min. Since Cry proteins are resistant to trypsin, this latter trypsinization enhanced the signal on western blot. Protein extracts were analyzed using SDS-PAGE and the subsequent immunoblot assay were performed according to a published method (Stewart et al., 1996). Forty micrograms of leaf protein extract and 60 μg of stem and root protein extracts were added to each well for analysis. The Cry2Aa2 protein used as a positive control was a wild-type protein isolated from *E. coli* (Clone ECE126, *Bacillus* Genetic Stock Center, USA) as described by Ge et al. (1990). The blot was incubated with rabbit anti-Cry2Aa2 serum, followed by incubation with goat anti-rabbit/alkaline phosphatase. The blot was exposed to nitroblue tetrazolium/bromochloroindolyl phosphate substrate to develop the signal. Protein expression was quantified by scanning densitometry and comparison to protein standards.

Insect bioassays

The egg masses of tobacco budworm, *Heliothis virescens,* were purchased from North Carolina State University (NCSU) Insectary, USA (http://www.cals.ncsu.edu:8050/entomology/insectary/). These eggs were hatched at 28°C for 24 h to obtain neonate larvae. Detached leaf insect bioassays were performed using a previously described method (Stewart et al., 1996). Leaves were detached from the plants and placed on moist filter papers in Petri dishes. Ten neonate larvae were placed on top of each excised tobacco leaf. Three leaves from each plant were used separately in this bioassay. The mortality of the larvae was counted 3 days after the infestation.

Artificial diet incorporation assays were performed to evaluate the toxicity of Cry2Aa2 in leaf, stem and roots of these plants. Artificial diet for *H. virescens* larvae was purchased from NCSU insectary and prepared according to the supplier's instructions. Lyophilized powder of plant leaves, stems and roots was added to the artificial diet in dilutions. The NCSU artificial diet for *H. virescens* was spiked with 10, 25, 50 and 100 mg of transgenic tissue powder. Five neonate larvae of *H. virescens* were placed in each vial containing 1 g of diet. Each assay was preformed in triplicate. For negative control, comparable quantities of lyophilized powder from non-transgenic tobacco leaf, stem, and root were added to the diet. Cry2Aa2 purified from *E. coli* clone ECE126, which was obtained from the *Bacillus* Genetic Stock Center (BGSC, USA), was used as a positive control. Mortality rate of larvae was monitored over 6 day periods. Larvae were recorded as dead when there was no movement upon touching.

Results

Screening for transgenic plants by PCR

Tobacco plants were transformed with the *cry2Aa2* expression cassette using *Agrobacterium*. The putative transgenic plants (kanamycin selection) were screened by PCR analysis using *cry2Aa2* sequence-specific primers. A total of 27 out of 30 tobacco plants examined were found positive for the presence of *cry2Aa2* sequence. Data from six representative transgenic plants are shown in Figure 2. The 0.8 kb PCR-amplified fragment was observed in the genomic DNA of all 27 transgenic plants. No amplification occurred from non-transformed tobacco DNA used as a negative control.

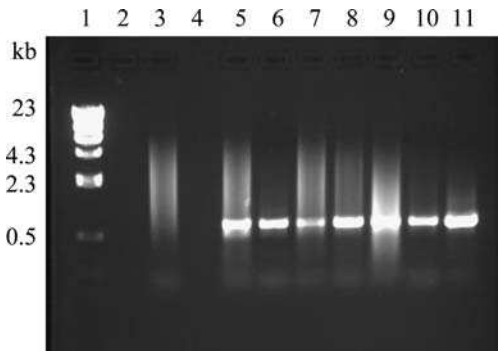

Figure 2. PCR amplification of *cry2Aa2* coding sequence in transgenic tobacco plants. Lane 1: λ/*Hin*dIII DNA marker; lanes 5–10: transgenic tobacco plants; lane 3: non-transgenic tobacco plant; lane 11: positive control (p2AST) (lanes 2 and 4 are empty).

Southern blot analysis

Southern blot analysis of four PCR-positive transgenic plants (T_0) was performed to verify site-specific integration of transgene and to establish transgene copy number. The four representative plants used in this analysis were 2AST(A), 2AST(E), 2AST(F) and 2AST(G). Initially, the restriction enzyme *Hin*dIII was used to asses the transgene copy number since this enzyme cuts only once within the T-DNA region of the expression cassette (Figure 1). The number of bands on the blot resulting from the DNA hybridization was assigned as the locus copy number for each transgenic plant. The transgenic plants 2AST(A), 2AST(E), 2AST(F) and 2AST(G) appeared to have seven, two, two and one transgene copies, respectively. The number of bands counted was however not accurate as many bands were compressed together and were thus difficult to count separately. To clarify the number of transgenic insertions in the plant genome, a second Southern blot was performed by digesting the plant genomic DNA with *Eco*RI. The *Eco*RI restriction site was located opposite to *Hin*dIII site in the expression cassette within the T-DNA region of p2AST (Figure 1). Upon this second analysis, the transgenic plant 2AST(A) was shown to contain eight transgene copies whereas the plants 2AST(E), 2AST(F) and 2AST(G) contained two, four and one transgene copies, respectively (Figure 3). The Southern analysis using two different restriction sites on the opposite ends of expression cassette in p2AST proved to be helpful in obtaining a more lucid picture of the copy number of the inserted transgene in the plant genome. The transgenic plant 2AST(A) which first appeared to have seven insertions showed eight copies of the *cry2Aa2* coding sequence. Similarly, 2AST(F) was found to have two additional insertions than was originally thought. The plants 2AST(E) and 2AST(G) continued to show two and one copies of the transgene, respectively.

Protein expression analysis

The Cry2Aa2 protein expression for the transgenic plants was measured through western blot analysis. The protein extracts of leaves and stems from all the transgenic plants displayed the expected 71 kDa size band which was absent in the non-transgenic plants (Figures 4 and 5). The

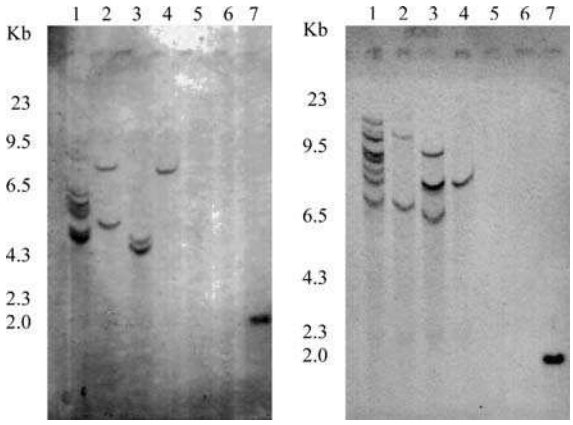

Figure 3. Southern blot analysis of transgenic tobacco plants' DNA probed with PCR-amplified sequence of *cry2Aa2* coding sequence from p2AST plasmid. Lanes 1–4: genomic DNA of transgenic tobacco plants 2AST(A), 2AST(E), 2AST(F) and 2AST(G), respectively; lane 5: genomic DNA of non-transgenic tobacco plant; lane 7: positive control (*cry2Aa2* coding sequence). Left panel: total genomic DNA digested with *Hin*dIII. Right panel: total genomic DNA digested with *Eco*RI.

levels of Cry2Aa2 expression were quantified by densitometric analysis of western blot using Alpha Imager (1220 v 5.04) software. The expression of Cry2Aa2 in 2AST(A), 2AST(E), 2AST(F) and 2AST(G) plants was estimated to be 0.16, 0.21, 0.12 and 0.03%, respectively, of total soluble leaf proteins. The amount of protein detected in the stems of 2AST(A), 2AST(E), 2AST(F) and 2AST(G) was 0.010, 0.012, 0.009 and 0.004%,

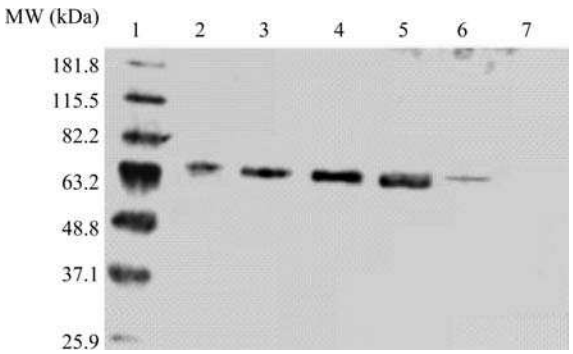

Figure 4. Detection of Cry2Aa2 protein in transgenic tobacco plants. Forty micrograms of total leaf protein extract was separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. The blot was probed with polyclonal antibody against Cry2A followed by goat anti-rabbit IgG. lane 1: Protein standard marker (Invitrogen); lane 2: Cry2A protein, positive control, isolated form *E. coli*, ECE126 (courtesy of BGSC); lanes 3–6: Transgenic tobacco plants, 2AST(A), 2AST(E), 2AST(F) and 2AST(G), respectively; lane 7: Non-transgenic tobacco plant (negative control).

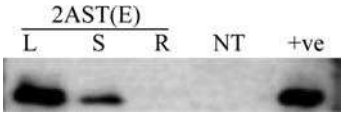

Figure 5. Tissue specificity of Cry2Aa2 expression in transgenic tobacco plants. Forty micrograms of leaf (L) protein extracts and 60 μg of stem (S) and root (R) protein extracts from the transgenic plants 2AST(E) were separated on 10% SDS-PAGE. The proteins were transferred to nitrocellulose membrane. The blot was probed with polyclonal antibody against Cry2A followed by goat anti-rabbit. NT, Leaf protein extract from non-transgenic tobacco plant; +ve, positive control, Cry2A isolated form *E. coli*, ECE126.

respectively, of total soluble stem proteins. The protein extracts from roots did not show the presence of Cry2Aa2 on immunoblot. In Figure 5 only the transgenic plant 2AST(E) is shown for comparison. Protein samples isolated from different stem sections of the same plant showed variable levels of Cry2Aa2 on western blot due to heterogeneity in green tissue content (data not shown).

Tissue difference in entomocidal activity

When the leaves from transgenic plants were fed to *H. virescens*, 100% mortality was observed between 36 and 72 h of the assay in all the representative transgenic plants 2AST(A, E, F and G). No evidence of mortality among the negative control larvae was observed, where the entire leaves were eaten (the leaves of R_0 and R_1 of the transgenic plant 2AST(A) are shown in Figure 6).

Stem cuttings are lignified and woody compared to tender leaves, so we resorted to preparing a lyophilized ground powder as a dry dietary supplement, in order to assess the entomocidal activity of transgenic stems. Artificial diet incorporation assayed the mortality of *H. virescens* larvae fed lyophilized leaf and root powder as well. The larval mortality using 10 mg of lyophilized stem powder of 2AST(A), 2AST(E), 2AST(F) and 2AST(G) was 41, 44, 39 and 22%, respectively (Table 1). Twenty-five milligrams of lyophilized stem powder of 2AST(A), 2AST(E), 2AST(F) and 2AST(G) killed 73, 89, 66 and 40% of the larvae, respectively. The mortality reached 100% from these plants at 50 mg of stem powder except the plant 2AST(G) which could kill 69% larvae. A mortality rate of 100% was observed from 100 mg stem powder of the plant 2AST(G). The lyophilized leaf powder of these transgenic plants killed 100% of the

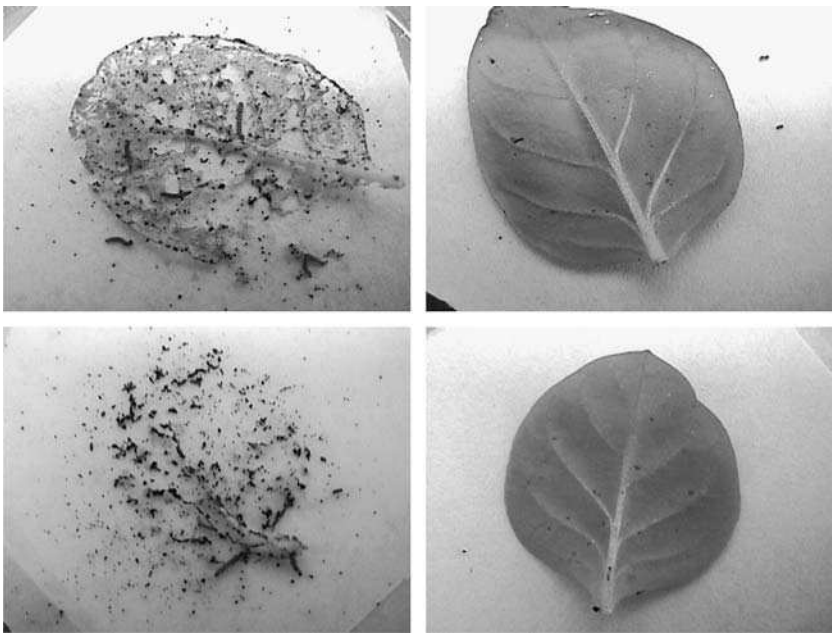

Figure 6. Detached leaf bioassay of transgenic tobacco against *H. virescens*. Upper left panel: leaf from non-transgenic plant, Upper right panel: leaf from R_0 transgenic plant 2AST(A). Lower left panel: leaf from non-transgenic plant, Lower right panel: leaf from R_1 transgenic plant 2AST(A). Ten neonate larvae were allowed to feed on each leaf. The assays were performed in triplicate (photographs were taken on day 4 of the assay).

Table 1. Mortality of *H. virescens* larvae to different doses of lyophilized leaf, stem and roots of transgenic tobacco plants

Dose mg/g diet	% Mortality ± SE														
	2AST(A)			2AST(E)			2AST(F)			2AST(G)			NT		
	L	S	R	L	S	R	L	S	R	L	S	R	L	S	R
10 mg	100 ±0	41 ± 1.73	2.9 ± 0.47	100 ±0	44 ± 2.16	2.3 ± 0.93	100 ±0	39 ± 1.08	1.8 ± 0.36	100 ±0	22 ± 3.69	2.3 ± 0.57	2.8 ± 0.34	3.0 ± 0.22	2.3 ± 0.62
25 mg	100 ±0	73 ± 4.4	4.8 ± 1.49	100 ±0	89 ± 4.3	3.9 ± 0.86	100 ±0	66 ± 3.36	2.9 ± 0.92	100 ±0	40 ± 3.81	4.5 ± 1.64	4.4 ± 1.79	4.9 ± 1.07	4.6 ± 1.9
50 mg	100 ±0	100 ±0	3.3 ± 0.87	100 ±0	100 ±0	3.8 ± 1.03	100 ±0	100 ±0	2.3 ± 0.5	100 ±0	69 ± 3.95	2.7 ± 1.79	4.7 ± 1.03	5.0 ± 1.91	3.6 ± 0.77
100 mg	100 ±0	100 ±0	4.9 ± 1.92	100 ±0	100 ±0	3.3 ± 1.1	100 ±0	100 ±0	3.4 ± 0.89	100 ±0	100 ±0	4.5 ± 1.28	2.0 ± 0.79	3.9 ± 1.57	4.9 ± 2.65

NT, non-transgenic tobacco plant; L, leaf; S, stem; R, root.

H. virescens larvae at all doses (10–100 mg) used. The larval mortality from the lyophilized root powder of this transgenic plant was only 1–5% which was similar to the lyophilized powder (leaf, stem or root) of non-transgenic plant.

Inheritance of transgenic trait

Seeds obtained from R_0 plants were used to study the inheritance of transgene transmission. R_1 plants were screened for the presence of the *cry2Aa2* gene by PCR (data not shown) and their leaves were used for bioassay studies. The results of these bioassays indicated that the second generation transgenic plants still provided the protection of 100% mortality against *H. virescens* larvae (the leaves of R_0 and R_1 of the transgenic plant 2AST(A) are shown in Figure 6).

Discussion

Insects can evolve resistance when exposed to Bt under constant selection pressure (Stone et al., 1989; Tabashnik et al., 1990; Meng et al., 2004). Insect resistance may become the largest impediment to the successful deployment of Bt crops. One effective way of lowering the selection pressure is by restricting the expression of Bt proteins to the tissues susceptible to insect damage (Gould, 1998; Frutos et al., 1999). To reduce the amount of active entomocide that might help select for resistance allele(s) in a pest population, we tested whether the ST-LS1 promoter from potato could limit the expression of the *cry2Aa2* coding sequence to green tissues in tobacco (*N. tabacum* cv. Xanthi).

The expression of the Cry2Aa2 protein in the leaves of individual transgenic plants was determined by immunoblot analysis. A band of 71 kDa corresponding to the purified Bt protein was detected in the protein samples of transgenic plants (Figure 4). The levels of Cry2Aa2 expression were between 0.03 and 0.21% of total soluble protein in the plants analyzed. The high expression levels of Bt protein reported here in the green tissues of a dicot plant are comparable to those reported by Datta et al. (1998) in monocot plants generated through nuclear transformation. The green tissue-specific expression of a Bt protein (Cry1C) has been previously reported in

tobacco by Christov et al. (1999) using the (*rbcS*) gene promoter. The ST-LS1 and *rbcS* genes belong to the same class of genes. They are nuclear genes encoding a chloroplast-located protein involved in photosynthesis (Stockhaus et al., 1989). The level of expression of Bt protein driven by the *rbcS* gene promoter was however low (0.02–0.03%).

We wanted to evaluate the effect of transgene copy number on the expression levels of Cry2Aa2. The transgenic plants 2AST(G, E, F and A) had one, two, four and eight copies of *cry2Aa2*, respectively. The coincidental presence of transgene(s) in this multiple order permits one to check for correlation between copy number and transgene expression levels. No correlation however, was found between the copy number and the expression levels of Cry2Aa2 in these plants. For example, the transgenic plant 2AST(E) containing two copies of *cry2Aa2* had the highest level of expression (0.21%) whereas the plants with four and eight insertions (2AST(F) and 2AST(A), respectively) had lower expression levels, 0.11 and 0.16% (Figure 7). The site of integration of a foreign gene in a plant genome plays a key role in determining the expression levels. This is referred to as position effect (Mlynarova et al., 1995; Maqbool & Christou, 1999). The absence of a correlation between the levels of expression and the transgene copy number might be the result of such a position effect phenomenon in the present study. Moreover, the T-DNA region may be associated with deletions or insertions such that it is not incorporated intact into the target genome (Gorbunova & Levy, 1997; Meza et al., 2002). The *cry2Aa2* containing T-DNA region in the present study might have integrated in a truncated form lacking the *Eco*RI or *Hin*dIII sites. As a result of the probable loss of T-DNA restriction sites, the number of bands on Southern blot is not accurate in representing the *cry2Aa2* insertions in the plant genome. A detailed study of the regions flanking the transgene insertions site in the plant genome might be helpful in correlating the expression levels and transgene copies in the present study.

The Cry2Aa2 protein produced in these plants appeared properly folded and functional. Detached leaf bioassay analysis indicated a 100% mortality of *H. virescens* larvae when these larvae were fed leaves from these transgenic plants. All the larvae on transgenic leaves were dead by day 4 of the bioassay, whereas the larvae that were exposed to non-transgenic tobacco leaves, survived (Figure 6).

Spatial control of insecticide deployment

We evaluated the relative amount of leaf, stem and root material required to cause mortality of *H. virescens* larvae. As leaf, stem and root tissues are not equally soft and tender for the insect larvae to feed on, we employed an artificial diet incorporation assay using lyophilized ground powders of these plant parts. For example, leaves from the transgenic plants 2AST(A, E, F and G) had Cry2Aa2 expression levels ranging from 0.03

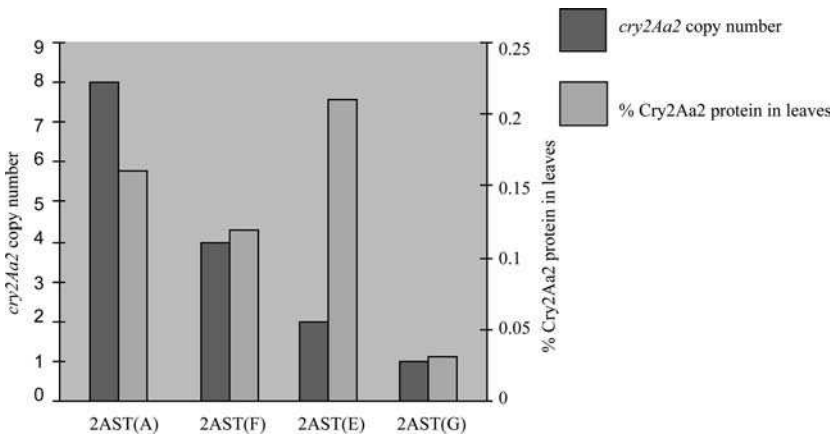

Figure 7. The relationship between Cry2Aa2 levels in the transgenic plants and the copy number of the c*ry2Aa2* transgene.

to 0.21% of total soluble protein. In all these plants, 10 mg lyophilized leaf powder per gram of artificial diet was sufficient to achieve 100% killing of the larvae (Table 1). Comparatively, when using stem cuttings, 50–100 mg of lyophilized stem powder from these plants was required to cause 100% mortality in these larvae. The increase in mortality rate using increased amounts of lyophilized stem powder from the same plant was proportional to the Cry2Aa2 levels in their stems. For example 10 mg of powder from the plant 2AST(G) with the lowest Cry2Aa2 levels in stem (0.004% of the total soluble stem proteins), had a mortality rate of only 22%. The mortality caused by the stem powder of this plant rose up to 66 and 100% when the amount of stem powder used was increased to 50 and 100 mg, respectively. A similar trend was observed for each plant: increasing the biomass of stem powder fed to *H. virescens* larvae lead to increased mortality. The quantity of freeze-dried powder required was proportional to the expression levels of Cry2Aa2 in each plant stem. Our results might suggest that the ST-LS1 promoter is more active in leaf than in stem. Alternatively, the ST-LS1 promoter might be equally active in stem and leaf tissue. The lower mortality rates from stem tissue may be a result of the number of 'green' cells actually expressing ST-LS1-*cry2Aa2* in each organ, given that photosynthetic tissues are restricted to stem epidermis. Such fresh weight protein accumulation comparisons await stricter cell counts (photosynthetic vs. non-green cells). Since the leaves and to some extent the stems of tobacco plants are green and are photosynthetic, Cry2Aa2 under the control of ST-LS1 gene promoter was produced only in these tissues and caused the mortality of *H. virescens*. This is further supported by the observation that no mortality occurred when lyophilized root powder of these plants was used. Root cells are devoid of chloroplasts hence no Cry2Aa2 was present to cause insect mortality. Immunoblot analysis of protein extracts from leaf, stem and root of the 2AST(A, E, F and G) plants (Figure 5) corroborates the conclusion that the ST-LS1 promoter is effective in driving Cry2Aa2 expression exclusively in green tissues.

Stable accumulation of the Cry2Aa2 protein strictly in the green tissues of the transgenic tobacco in our study validates the use of the ST-LS1 gene promoter for a successful localized deployment of Bt protein in crop plants. The addition of the ST-LS1 gene promoter to the all too short list of leaf specific promoters from and for dicot protection is a major step forward in the Insect Resistance Management (IRM) of Bt crops. The restriction of Bt expression to the green tissues of the plant may provide larger refuges for susceptible alleles by preventing the exposure of insect populations to constant selection pressure. The tissue specific expression of Bt toxins in transgenic plants is an important technological approach in sustaining Bt's effectiveness for controlling insects, particularly those that feed by boring into plant tissues. For example, cowpea pod borer, *Maruca vitrata* (Fab. syn. *M. testulalis* Geyer), enters the flower buds or pods by tunneling into them (Sharma, 1998). Targeted expression of Cry protein strictly to the green tissues might help ensure compliance with the IRM guidelines, an effective control of such insects in a sustainable manner.

Acknowledgements

This work was funded by The Rockefeller Foundation and NSERC. MM is grateful for a UNESCO fellowship. We are thankful to Menachem Keller, Vitality Biotechnologies Inc., Israel, and the *Bacillus* Genetic Stock Center, USA, for providing us the ST-LS1 gene promoter and the *cry2Aa2* clone in *E. coli* (ECE126), respectively.

References

Albani D, Sardana R, Robert LS, Altosaar I, Arnison PG and Fabijanski SF (1992) A *Brassica napus* gene family which shows sequence similarity to ascorbate oxidase is specifically expressed in developing pollen. Molecular characterization and analysis of promoter activity in transgenic tobacco plants. *Plant J* **2**: 332–343.

Alinia F, Ghareyazie B, Rubia L, Bennett J and Cohen MB (2000) Effect of plant age, larval age, and fertilizer treatment on resistance of *cry1Ab*-transformed aromatic rice to lepidopterous stem borers and foliage feeders. *J Econ Entomol* **93**: 484–493.

Christov NK, Imaishi H and Ohkawa H (1999) Green-tissue-specific expression of a reconstructed *cry1C* gene encoding the active fragment of *Bacillus thuringiensis* delta-endotoxin in haploid tobacco plants conferring resistance to *Spodoptera litura*. *Biosci Biotechnol Biochem* **63**: 1433–1444.

Crickmore N, Zeigler DR, Feitelsson J, Schnepf E, Van Rie J, Lereclus D et al. (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev* **62**: 807–813.

Datla RSS, Hammerlindl JK, Panchuk B, Pelcher LE and Keller W (1992) Modified binary plant transformation vectors with the wild-type gene encoding NPTII. *Gene* **122**: 383–384.

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N (1998) Constitutive and tissue-specific differential expression of the *cryIA(b)* gene in transgenic rice plants conferring resistance to rice insect pest. *Theor Appl Genet* **97**: 20–30.

Frutos R, Rang C and Royer M (1999) Managing insect resistance to plants producing *Bacillus thuringiensis* toxins. *Crit Rev Biotechnol* **19**: 227–276.

Ge AZ, Pfister RM and Dean DH (1990) Hyperexpression of a *Bacillus thuringiensis* δ-endotoxin encoding gene in *Escherichia coli*: properties of the product. *Gene* **93**: 49–54.

Gorbunova V and Levy AA (1997) Non-homologous DNA end joining in plant cells is associated with deletions and filler DNA insertions. *Nucl Acids Res* **25**: 4650–4657.

Gould F (1998) Sustainability of transgenic insecticidal cultivars: Integrating pest genetics and ecology. *Ann Rev Entomol* **4**: 3701–3726.

High SM, Cohen MB, Shu QY and Altosaar I (2004) Achieving successful deployment of Bt rice. *Trends Plant Sci* **9**: 286–292.

Horsch RB, Fry JE, Hoffmann NL, Eichholtz D, Rogers SG and Fraley RT (1985) A simple and general method for transferring genes into plants. *Science* **227**: 1229–1231.

James C (2003) Global Status of Commercialized Transgenic Crops: ISAAA Briefs No. 30. ISAAA, Ithaca, NY.

Maqbool SB and Christou P (1999) Multiple traits of agronomic importance in transgenic indica rice plants: analysis of transgene integration patterns, expression levels and stability. *Mol Breed* **5**: 471–480.

Maqbool SB, Husnain T, Riazuddin S, Masson L and Christou P (1998) Effective control of yellow stem borer and rice leaf folder in transgenic rice indica varieties Basmati 370 and M 7 using the novel δ-endotoxin *cry2A Bacillus thuringiensis* gene. *Mol Breed* **4**: 501–507.

Meng F, Shen J, Zhou W and Cen H (2004) Long-term selection for resistance to transgenic cotton expressing *Bacillus thuringiensis* toxin in *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae). *Pest Manage Sci* **60**: 167–172.

Meza TJ, Stangeland B, Mercy IS, Skarn M, Nymoen DA, Berg A et al. (2002) Analyses of single-copy *Arabidopsis* T-DNA-transformed lines show that the presence of vector backbone sequences, short inverted repeats and DNA methylation is not sufficient or necessary for the induction of transgene silencing. *Nucl Acids Res* **30**: 4556–4566.

Mlynarova L, Jansen RC, Conner AJ, Stiekema WJ and Nap JP (1995) The MAR-mediated reduction in position effect can be uncoupled from copy number-dependent expression in transgenic plants. *Plant Cell* **7**: 599–609.

Naimov S, Dukiandjiev S and de Maagd RA (2003) A hybrid *Bacillus thuringiensis* delta-endotoxin gives resistance against a coleopteran and a lepidopteran pest in transgenic potato. *Plant Biotechnol J* **1**: 51–57.

Nester EW, Thomashow LS, Matthew M and Gordon M (2002) 100 Years of *Bacillus thuringiensis*: a critical scientific assessment. American Academy of Microbiology, 1752 N Street, NW Washington, DC, 16 pp.

Ramesh S, Nagadhara D, Pasalu IC, Kumari AP, Sarma NP, Reddy VD et al. (2004) Development of stem borer resistant transgenic parental lines involved in the production of hybrid rice. *J Biotechnol* **111**: 131–141.

Sambrook J and Russell DW (2001) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Sharma HC (1998) Bionomics, host plant resistance, and management of the legume pod borer, *Maruca vitrata* – a review. *Crop Protect* **17**: 373–386.

Shu Q-Y, Ye G-Y, Cui H-R, Cheng X, Xiang Y, Wu D et al. (2000) Transgenic rice plants with a synthetic *cry1Ab* gene from *Bacillus thuringiensis* were highly resistant to eight lepidopteran rice pest species. *Mol Breed* **6**: 433–439.

Stewart CN Jr, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D et al. (1996) Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis cryIAc* gene. *Plant Physiol* **112**: 121–129.

Stockhaus J, Eckes P, Rocha-Sosa M, Schell J and Willmitzer L (1987) Organ-specific and dosage-dependent expression of a leaf/stem specific gene from potato after tagging and transfer into potato and tobacco plants. *Nucl Acids Res* **15**: 3479–3491.

Stockhaus J, Schell J and Willmitzer L (1989) Identification of enhancer elements in the upstream region of the nuclear photosynthetic gene *ST-LSI*. *Plant Cell* **1**: 805–813.

Stone TB, Sims SR and Marrone PG (1989) Selection of tobacco budworm for resistance to a genetically engineered *Pseudomonas fluorescens* containing the δ-endotoxin of *Bacillus thuringiensis* subsp. *kurstaki*. *J Invertebr Pathol* **53**: 228–234.

Tabashnik BE, Cushing NL, Finson N and Johnson MW (1990) Field development of resistance to *Bacillus thuringiensis* in diamondback moth (Lepidoptera: Plutellidae). *J Econ Entomol* **83**: 1671–1676.

Toenniessen GH, O'Toole JC and DeVries J (2003) Advances in plant biotechnology and its adoption in developing countries. *Curr Opin Plant Biol* **6**: 191–198.

van Voorthuysen T, Regierer B, Springer F, Dijkema C, Vreugdenhil D and Kossmann J (2000) Introduction of polyphosphate as a novel phosphate pool in the chloroplast of transgenic potato plants modifies carbohydrate partitioning. *J Biotechnol* **77**: 65–80.