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Transgenic broccoli with high levels of *Bacillus thuringiensis* Cry1C protein control diamondback moth larvae resistant to Cry1A or Cry1C

Jun Cao[1], Juliet D. Tang[2], Nicolai Strizhov[3], Anthony M. Shelton[2] and Elizabeth D. Earle[1,*]
[1]*Department of Plant Breeding, Cornell University, Ithaca, NY 14853, USA (*author for correspondence; e-mail ede3@cornell.edu);* [2]*Department of Entomology, Cornell University, New York State Agricultural Experiment Station, Geneva, NY 14456, USA;* [3]*Max-Planck-Institute für Züchtungsforschung, Carl-von-Linné-Weg 10, 50829 Koln, Germany*

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Abstract

A synthetic *Bacillus thuringiensis* (Bt) *cry1C* gene was introduced into broccoli (*Brassica oleracea* ssp. *italica*) by *Agrobacterium*-mediated transformation. Twenty-one Cry1C transgenic plants were regenerated from 400 hypocotyl and petiole explants. Variable amounts of stable steady-state *cry1C* mRNA accumulated in different transgenic plants. Cry1C protein (up to 0.4% of total soluble protein) was produced in correlation with the *cry1C* mRNA levels. Leaf section and whole-plant bioassays were done using diamondback moth (DBM) larvae from lines susceptible to Bt or resistant to Cry1A or Cry1C proteins ($Cry1A^R$ or $Cry1C^R$, respectively). Plants with high levels of Cry1C protein caused rapid and complete mortality of all three types of DBM larvae with no defoliation. Plants with lower levels of Cry1C protein showed an increasing differential between control of susceptible of $Cry1A^R$ DBM. This study demonstrated that high production of Cry1C protein can protect transgenic broccoli not only from susceptible or $Cry1A^R$ DBM larvae but also from DBM selected for moderate levels of resistance of Cry1C. The Cry1C-transgenic broccoli were also resistant to two other lepidopteran pests of crucifers (cabbage looper and imported cabbage worm). These plants will be useful in studies of resistance management strategies involving multiple transgenes.

Introduction

Genetic engineering of crops with insecticidal endotoxin genes isolated from *Bacillus thuringiensis* (Bt) has launched a new era in crop protection. Several Bt toxin genes have been cloned and transferred to various crop plants for insect control. Bt-transgenic cotton [17], potato [1], and maize [6] have proved to be highly effective in controlling major lepidopteran and coleopteran pests and are now commercially available in the USA [7]. Because insects have demonstrated a high capacity to develop resistance to a wide array of chemical insecticides, the possibility that insects might evolve resistance to Bt-transgenic plants is a serious concern. Bt crops provide a constant selection pressure on insect populations, which may accelerate the development of resistant insect populations. To ensure durability of Bt-transgenic crops, appropriate resistance management strategies must be developed.

Evolution of resistance to Bt in field populations of the diamondback moth (DBM, *Plutella xylostella*), a major crucifer vegetable pest, has been documented in Hawaii [30], Japan [5], Florida [27], Central America [15], and China [37] after extensive use of sprays containing Bt subsp. *kurstaki*. Resistance in these colonies of *P. xylostella* has been reported to be controlled by one or few autosomal genes [5, 31, 33] and was attributed to high levels of tolerance to the insecticidal Cry1A(a), Cry1A(b), and Cry1A(c) proteins [31, 34]. The colonies showed cross resistance to Cry1F and Cry1J [31, 32] but little cross-resistance to other toxin families such as Cry1B, Cry1C, and Cry1D [31, 34].

The imminence of resistance to other toxins is further demonstrated by the first reports of moderate levels of resistance to Cry1C now being publicized as growers rely more heavily on Cry1C-containing formulations of Bt insecticides [10]. This colony, collected from Hawaii, showed about 20-fold levels of resistance to Cry1C toxin. DBM larvae with resistance to Cry1C have also been selected from a population collected in South Carolina where the starting level of resistance after collection from the field was about 30-fold (Tang, unpublished data).

Regeneration of transgenic *Brassica oleracea* has been achieved through *Agrobacterium tumefaciens*-mediated transformation [3, 35]. Infection of peduncle, hypocotyl, or petiole explants with *A. tumefaciens* carrying a *cry1A(c)* gene has produced large numbers of transgenic broccoli plants [12]. Many of these plants caused 100% mortality of first instar larvae of Bt-susceptible DBM as well as the F_1 progeny of the cross between the susceptible and Cry1A^R colonies [13]. However, Cry1A^R DBM larvae survived on the transgenic plants [13].

Field derived Bt-resistant DBM populations and the ability to produce *B. oleracea* carrying Bt toxin genes provide an excellent model system for the study of interactions between insects and transgenic plants and permit tests of various strategies for delaying the development of resistant insect populations. Such studies could also provide a better understanding of the dynamics of evolution of Bt-resistant insects and the genetics and biochemical mechanisms of insect resistance.

The objective of our study was to obtain broccoli plants producing Cry1C protein and to assess the ability of these plants to control various strains of DBM. We report the introduction and high expression of a synthetic *cry1C* gene [29] in broccoli and show that plants with a high level of Cry1C protein cause rapid and complete mortality of neonate and older larvae of susceptible, Cry1A^R, and Cry1C^R DBM.

Materials and methods

Transformation vector

Transformation of broccoli was performed with a binary vector as shown in Figure 1. The vector contained a synthetic *cry1C* gene (GenBank accession number X99103) driven by a CaMV 35S promoter with four enhancer regions and a hygromycin resistance gene as the selective marker. The synthesis of the *cry1C* gene was based on the sequence of the corresponding wild-type gene (*cry1Ca5*; GenBank accession number X96682) (28, 29). About 15% of the *cry1C* gene was modified to remove cryptic polyadenylation sites, potential splicing sequences, and RNA instability sequence motifs, as well as to change the codon usage to fit plant codon preference [28].

Insects

Strains

Three populations of DBM larvae were used in bioassays. The susceptible colony was collected in 1988 from cabbage in Geneva, NY, and reared continuously on a wheat germ casein artificial diet for more than 170 generations [26]. The Cry1A^R colony was collected in 1994 from commercial crucifer fields in Loxahatchee, Florida and reared on Cry1A(c) expressing broccoli [13] for more than 34 generations. Compared to the LC_{50} of the susceptible colony, the Cry1A^R colony was 829-fold resistant to Javelin, a commercial formulation of Bt ssp. *kurstaki* [16]. The Cry1C^R colony was collected in 1997 from Lexington, SC. After field collection and prior to laboratory selection, larvae from the Cry1C^R DBM were 34-fold resistant to Cry1C protein (Tang, unpublished).

Cry1C selection of insects

Cry1C protoxin was obtained from W. Moar, Auburn University, Alabama. The *cry1C* gene, which was from Bt ssp. *entomocidus*, was expressed in an acrystalliferous strain of HD-1 [14]. Beginning with the second generation after the Lexington colony was brought in from the field, Cry1C^R larvae were selected every generation with increasing concentrations of purified Cry1C protoxin. In the selection bioassay, larvae were allowed to feed for 72 h on leaves that had been dipped for 10 s in solutions of Cry1C. Surviving larvae were then transferred to clean oilseed rape plants where they completed development. For these experiments, the Cry1C^R colony had been selected six times. For the first, second, and last selections, we used Cry1C concentrations of 6, 100, and 250 μg/ml, which produced mortality of 34.8, 82.8, and 69.9%, respectively. After the 6th laboratory selection, resistance at the LC^{50} for the Cry1C^R colony had increased an additional 4-fold compared to the level seen before selection in the laboratory (Tang, unpublished). The insects used in bioassays of transgenic broccoli plants thus had over 100-fold resistance to Cry1C protein.

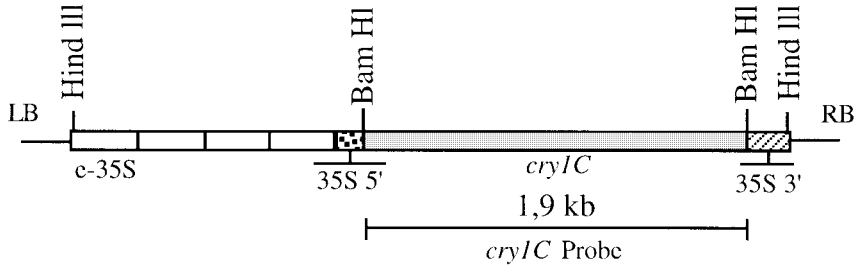

Figure 1. The binary vector pNS_6 used in broccoli transformation. Expression of the *cry1C* gene is regulated by the CaMV35S promoter (35S 5′) with four repeats of the upstream enhancer region (−90 to −418; e-35S) and terminated by the polyadenylation sequence from the 35S RNA gene of CaMV (35S 3′). A 20 bp region from the 3′ end of the untranslated Ω leader sequence of tobacco mosaic virus RNA is located upstream of the coding sequence of the *cry1C* gene. Two *Bam*HI sites flanking the *cry1C*-coding region were used for DNA digestion in DNA blot hybridization and generation of the *cry1C* fragment used as a probe for DNA and RNA blot hybridization.

Broccoli transformation

Preculture of hypocotyl and petiole explants and cocultivation of explants with *A. tumefaciens* strain ABI harboring pNS_6 (Figure 1) were performed according to the method described [12]. Explants were then transferred to a modified regeneration medium [12] containing 2 mg/l BA, 0.2 mg/l NAA and 300 mg/l Timentin (Smithkline Beecham) and cultured for 7 days to eliminate *Agrobacterium*. For initial selection of transformed cells, explants were transferred to the same medium plus 10 mg/l hygromycin. Explants were maintained on this medium for 4 weeks with a subculture every two weeks. Emerging shoots as well as explants with green calli were subsequently transferred to regeneration medium containing only 2 mg/l BA in addition to 10 mg/l hygromycin and further cultured for 4 to 6 weeks. Hygromycin-resistant shoots were transferred to rooting medium [12] containing 10 mg/l hygromycin. Rooted transgenic plants were transplanted into soil mix as described [12].

Analysis of transgenic plants

PCR and DNA gel blot analysis

Total cellular DNA was isolated from leaf tissues as previously described [2]. Polymerase chain reaction (PCR) analysis of genomic DNA of transgenic plants was carried out with Taq polymerase (Boehringer-Mannheim) using the following primers for amplification of a 1.0 kb fragment from the *cry1C* gene: 5′ primer, 5′-GGAGAAAGATGGGGATTG-3′; 3′ primer, 5′-AACTCGTGCATCCCTACT-3′. PCR was carried out in a Perkin Elmer DNA Thermal Cycler. Temperature profiles of the hot-start cycle were 94 °C for 3 min; subsequently, 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min for 30 cycles, followed by a final extension cycle at 72 °C for 10 min. For Southern blot analysis, plant DNA digested with *Bam*HI was electrophoresed in 1% agarose gels and transferred onto nylon membrane. Blots were probed with a ^{32}P-labeled *Bam*HI-cleaved 1.9 kb DNA fragment covering the entire coding region of the *cry1C* gene in pNS_6 (Figure 1).

RNA gel blot analysis

Total RNAs were extracted from the leaf tissue following the protocol of Verwoerd *et al.* [36]. Northern analysis of the RNA samples was carried out following the protocol of Sambrook *et al.* [25]. RNAs (20 μg) were separated on agarose-formaldehyde gels and blotted onto nylon membrane. The probe used for RNA gel blot hybridization was a 1.9 kb *cry1C* fragment of pNS_6. The same blots were also probed with a 1.5 kb actin gene from *Brassica*. Relative amounts of mRNA bands were quantified by using a phosphorimager (Molecular Dynamics) and Image Quant V.3 software.

Imunoblotting

Protein was extracted in SDS sample buffer as described [25]. Briefly, fresh leaf tissue from individual transgenic plants was ground in liquid nitrogen with a pestle and homogenized in 200 μl of SDS sample buffer (100 mM Tris-HCl pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol). After heating for 3 min at 100 °C, the samples were centrifuged to remove insoluble materials. Protein concentrations in the supernatant were determined using a Lowry protein assay [8]. For immunoblots, protein extracts were electrophoresed in 10% SDS-polyacrylamide gels as described by Sambrook *et al.* [25]) and electro-transferred onto nitrocellulose. Immunoblotting was performed according to the ECL western blotting protocols (Amersham Life

Science). The primary antibody was raised against a truncated Bt ssp. *aizawai* Cry1C protein carrying 756 N-terminal amino acid (28) and diluted at 1:2000 for probing. Cry1C protein was quantified on the immunoblots by comparison of experimental samples with truncated Cry1C protein expressed and purified from *Escherichia coli*.

Insect bioassays

Detached leaf bioassays were performed using neonate, second-, third-, and fourth-instar larvae of susceptible, Cry1A^R, or Cry1C^R DBM. A leaf of each independent transgenic plant was cut into three sections. Each leaf section was used for bioassay of one type of DBM. For assays using neonates, a piece of aluminum foil with 10 eggs was placed on the surface of a leaf section. For assays using second, third, or fourth instar larvae, susceptible, Cry1A^R, and Cry1C^R larvae were grown on separate rapeseed plants prior to the assay. Five larvae of a selected developmental stage were placed on the surface of leaf sections. Each leaf section was placed in a baby food jar containing 1% water agar and maintained at 25 °C under a 16/8 h light/dark regime. All insect bioassays were performed in duplicate or triplicate. Leaf damage (estimated visually) and mortality of larvae were scored after 6 days (neonates) or 4 days (second- to fourth-instar stages). For insect assays with whole plants, separate plants of transgenic lines with various levels of Cry1C protein and control plants were infested with 80–100 late second instar larvae of susceptible, Cry1A^R, or Cry1C^R DBM, respectively. Leaf damage and insect mortality were evaluated after 5 days. Control of *Pieris rapae* (imported cabbage worm) and *Trichoplusia ni* (cabbage looper) by the transgenic plants was examined using neonate larvae in a detached leaf bioassay. Ten *P. rapae* or five *T. ni* larvae were placed on leaf sections from each transgenic plant. Leaf damage and insect mortality were scored after six days.

Results

Regeneration of transgenic broccoli plants

A synthetic *cry1C* gene [29] was introduced into commercial broccoli through *Agrobacterium*-mediated transformation. Initial selection of transgenic shoots was carried out on a modified regeneration medium [12] containing NAA (0.2 mg/l) as well as BA (2 mg/l) and hygromycin (10 mg/l); this allowed transformed cells to proliferate while differentiating to form shoots. After 2 weeks on this medium, shoots and calli derived from untransformed cells gradually turned brown. Green shoots and explants with green calli were then transferred to selective regeneration medium containing BA (2 mg/l) alone.

From 400 hypocotyl and petiole explants 21 hygromycin-resistant plants were recovered and transferred to soil.

PCR and Southern analyses

Hygromycin-resistant plantlets were screened for the presence of the *cry1C* gene using PCR technology with specific primers targeting the coding region of the gene. The expected 1.0 kb *cry1C* fragment was amplified from genomic DNA isolated from all putative transgenic plants but not from untransformed controls grown from seeds (data not shown). Subsequently, DNA isolated from transgenic broccoli plants was subjected to Southern analysis to provide additional evidence of integration of the *cry1C* gene into the broccoli genome. All transgenic plants analyzed gave the expected 1.9 kb band when genomic DNA was digested with *Bam*HI and probed with the coding sequence of the *cry1C* gene (Figure 2). Most transgenic plants (78%) contained a single copy of the *cry1C* gene. Two carried three copies, with one copy showing rearrangement of the gene. High-molecular-weight bands were observed when undigested genomic DNA was analyzed, further indicating the integration of the *cry1C* gene (data not shown).

Expression of the cry1C *gene in transgenic broccoli plants*

To examine the expression of the *cry1C* gene in independent transgenic plants, total RNAs isolated from leaf tissues were hybridized with the coding region of the synthetic *cry1C* gene. A sample of the RNA gel blot hybridization is shown in Figure 3. RNA gel blot analyses showed the expected 1.9 kb *cry1C* mRNA band in all transgenic plants analyzed except H19. Probing of the same blot with a *Brassica* actin gene showed that the amount of RNA applied was about the same for each plant. However, the accumulation of stable steady-state *cry1C* mRNA varied among the transgenic plants. As shown in Figure 3, line H14 produced the highest levels of *cry1C* mRNA while line H19 had the poorest expression. Most other transgenic lines (e.g., H10, H17) produced moderate

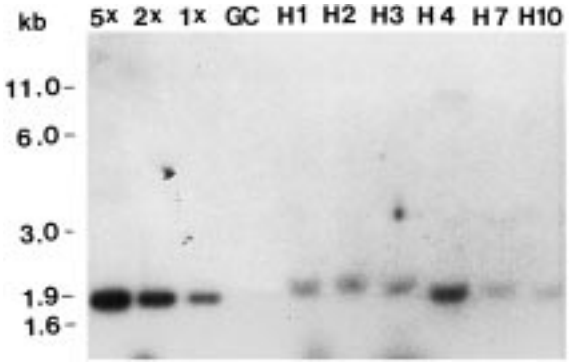

Figure 2. Southern blot analysis of transgenic broccoli plants. DNA was digested with *Bam*HI, and 10 μg of DNA was used for each lane. Lanes 1, 2 and 3 show a 1.9 kb *Bam*HI fragment isolated from plasmid pNS_6. 5×, 2× and 1× pNS6 plasmid DNA represent 5, 2 and 1 genome equivalents of DNA relative to 10 μg of broccoli genomic DNA, respectively. Lane 4 (GC) is an untransformed control. Lanes 5–10 are independent transgenic plants containing the integrated 1.9 kb fragment of the *cry1C* gene.

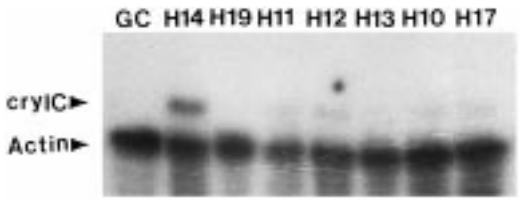

Figure 3. Northern blot analysis of *cry1C* mRNA in pNS_6 transgenic broccoli plants. For each line, 20 μg total RNA was fractionated on a formaldehyde-containing agarose gel and blotted to membrane. The membrane was hybridized with a ^{32}P-labeled 1.9 kb *Bam*HI fragment from pNS_6 covering the entire *cry1C* gene or with a 1.5 kb *Brassica* actin gene. Lanes: 1, RNA from untransformed broccoli; 2–8, RNA from transgenic plants. The arrow indicates the position of the hybridizing *cry1C* mRNA.

levels of the *cry1C* transcript. Relative differences in the mRNA signals on the northern blot were quantified with a phosphorimager (Molecular Dynamics). The level of *cry1C* mRNA in line H14 was estimated as about 50-fold higher than line H19.

Immunoblotting assays

To ascertain the levels of Cry1C protein in the transgenic plants, immunoblot analysis was performed with extracts from leaf tissues. As shown in Figure 4, H14 consistently produced highest levels of Cry1C protein. By comparing band intensities with those of known amounts of purified Cry1C protein, we estimated that Cry1C was ca. 0.4% of total soluble protein present in the leaves of the highest-expressing transgenic plants. Plants with moderate expression of the *cry1C* gene produced detectable but less Cry1C protein. Line H19,

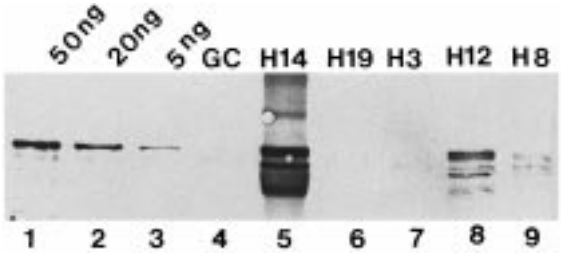

Figure 4. Western blot analysis of leaf tissue from transgenic broccoli expressing the *cry1C* gene. Lanes: 1, 2 and 3, 50, 20, or 5 ng respectively of Cry1C protein purified from *E. coli*; 4, proteins from untransformed broccoli; 5 to 9, proteins from transgenic plants expressing Cry1C protein. A total of 50 μg of soluble plant proteins was loaded per lane.

which had a very low level of *cry1C* mRNA, showed little or no Cry1C protein. Among the 19 transgenic plants tested were four with high protein levels, six with moderate levels, eight with low levels, and one with a very low level.

Insect bioassay

Leaf section testing

Transgenic plants carrying the *cry1C* gene were first tested for insect resistance by exposing leaf sections to neonates (Table 1). Neonates of susceptible, $Cry1A^R$ or $Cry1C^R$ DBM fed voraciously on untransformed controls, causing 65 to 85% defoliation, and grew to the second instar stage by day 6 after hatching from eggs. In contrast, susceptible, $Cry1A^R$ and $Cry1C^R$ neonates placed on transgenic leaves with high to moderate levels of Cry1C production stopped feeding, and most leaf parts remained unaffected (0% to 3% defoliation). Transgenic plants with low or very low Cry1C production showed 2% to 5% defoliation by susceptible and $Cry1A^R$ larvae and 5 to 25% by $Cry1C^R$ larvae.

Further leaf section bioassays used older larvae (second, third, and fourth instar) of susceptible, $Cry1A^R$, $Cry1C^R$, DBM to determine whether these would distinguish the bioactivity of the Cry1C protein in different transgenic broccoli plants (Table 1). Four days after the initial infestation, extensive feeding damage was observed on untransformed leaves, actively feeding larvae were visible on the damaged leaves, and there was no mortality (Figure 5). Transgenic plants with high Cry1C production showed no leaf damage and caused mortality of all three types of larvae within two to three days. On transgenic plants with moderate expression of the *cry1C* gene, only very small holes from early feeding were detected. Growth

Table 1. Defoliation of leaf sections of transgenic broccoli by diamondback moth larvae of different developmental stages after 6 days (neonates) or 4 days (older larvae).

Level of Cry1C protein	Number of plants tested	Larval stages	% defoliation caused by larvae		
			Susceptible	$Cry1A^R$	$Cry1C^R$
None		Neonate	85	70	65
		2nd, 3rd, and 4th instar	75–90	70–80	65–85
Very low	1	Neonate	5	3	25
		2nd, 3rd, and 4th instar	20	20	30
Low	8	Neonate	3–5	2–3	5–10
		2nd, 3rd, and 4th instar	3–10	5–10	10–20
Moderate	6	Neonate	0	0	1–3
		2nd, 3rd, and 4th instar	1–2	1–3	1–10
High	4	neonate	0	0	0
		2nd, 3rd, and 4th instar	0	0	0

of larvae on these plants was severely inhibited, and they died by day four after infestation. Older larvae caused more damage on leaf sections from plants with lower levels of Cry1C production (Table 1), with up to 30% defoliation by $Cry1C^R$ DBM.

Whole-plant testing

To assess the effectiveness of the Cry1C protein in whole plants, transgenic plants with high, moderate and low toxicity identified from the leaf assays were infested with late second instar larvae of susceptible, $Cry1A^R$ or $Cry1C^R$ DBM under high insect pressure (Table 2). After five days, untransformed control plants were severely to completely defoliated (Figure 6). Surviving larvae advanced to fourth instar, and some had pupated. Average body weight per larva increased by 2–3 mg. In contrast, high expression transgenic plants showed little or no feeding damage and produced 100% mortality of all three types of larvae. Transgenic plants with moderate production of Cry1C protein exhibited high toxicity to susceptible and $Cry1A^R$ larvae; however, they suffered slight leaf damage (3–5%) by $Cry1C^R$ larvae. Although a few $Cry1C^R$ larvae survived on these plants until day five, the size of the larvae basically remained unchanged. Line H3, producing a low level of Cry1C protein, conferred partial resistance: growth and development of larvae on this plant were severely inhibited. Line H19, which produced a very low level of *cry1C* mRNA and little or no Cry1C protein, suffered moderate defoliation (10 or 20%) by susceptible or $Cry1A^R$ larvae, respectively, and exhibited moderate inhibition of growth and development of these two types of larvae. In contrast, $Cry1C^R$ DBM caused severe leaf damage (90% defoliation) on line H19. Most $Cry1C^R$ larvae grew vigorously on this plant and advanced to fourth instar; some had pupated.

We also tested the insecticidal activity of the Cry1C-expressing plants with neonates of two other lepidopteran pests of crucifers, cabbage looper and imported cabbage worm. In leaf section bioassays transgenic plants with high or moderate expression of Cry1C protein caused rapid and complete mortality of both species with no defoliation.

Discussion

The protocol used for transformation of broccoli was modified from our previous work [12, 13]. Addition of NAA to the regeneration medium during the early selection process allowed transformed cells to proliferate while differentiating to form shoots and multiple green calli from which the transgenic shoots were

Table 2. Control of late second instar susceptible, $Cry1A^A$ and $Cry1C^{R*}$ diamondback moth larvae by transgenic broccoli plants after 5 days.

Level of Cry1C protein	Plant line	Type of larvae	Number of Larvae	Larval mortality (%)	Estimated defoliation (%)
None	Control	susceptible	99	0	90
		Cry1A-R	86	0	90
		Cry1C-R	86	0	90
Very low	H-19	susceptible	88	84	10
		Cry1A-R	85	82	20
		Cry1C-R	92	13**	90
Low	H-3	susceptible	75	93	5
		Cry1A-R	72	100	5
		Cry1C-R	70	89	15
Moderate	H-8	susceptible	84	100	<1
		Cry1A-R	72	100	<1
		Cry1C-R	72	97***	3–5
	H-9	susceptible	101	100	<1
		Cry1A-R	70	100	<1
		Cry1C-R	84	98***	3–5
High	H-14	susceptible	98	100	<1
		Cry1A-R	74	100	<1
		Cry1C-R	78	100	<1

*$Cry1C^R$ DBM were about 100-fold resistant compared to the susceptible colony.
**The suriving larvae continued to grow.
***The larvae that were still alive after 5 days failed to grow and died soon after.

recovered. Hygromycin was a more effective selective agent than kanamycin. Hygromycin at 10 mg/l completely inhibited growth and development of untransformed cells; explants with untransformed shoots and calli rapidly turned brown. Unlike kanamycin, hygromycin allowed proliferation and differentiation of only transformed cells, as seen by the fact that all plants recovered were transgenic.

High expression of Bt toxin genes has been achieved by optimizing expression vectors and modifying sequences of genes. The synthetic *cry1C* gene used [29] was modified to remove cryptic polyadenylylation sites, aberrant splicing sequences, and RNA instability motifs, as well as to change the codon usage to fit plant codon preference. For efficient transcription and translation, it was linked to an upstream untranslated leader of tobacco mosaic virus RNA under the control of the CaMV 35S promoter with four repeats of the upstream enhancer elements [29]. Optimized expression of the synthetic *cry1C* gene in alfalfa and tobacco resulted in the production of 0.01–0.2% of total soluble protein as Cry1C toxin and provided protection against Egyptian cotton leaf worm (*Spodoptera littoralis*) and the beet armyworm (*Spodoptera exigua*) [29]. Using the same construct in transgenic broccoli plants, we achieved the production of Cry1C protein up to an estimated level of 0.4% of total soluble protein. The levels of the Cry1C protein in our plants are consistent with the levels of expression of other modified Bt toxin genes in potato and tobacco [18, 19]. The range of the expression levels in independent transformants has been attributed to position effects and is not correlated with gene copy number or alteration of introduced genes [18].

Strong correlations were seen between the level of the *cry1C* mRNA, the production of Cry1C protein, and the insect control achieved. Transgenic broccoli plants with higher expression of *cry1C* mRNA produced more Cry1C protein and provided increased protection from DBM. Leaf and plant bioassays indicated that all plants with a high level of Cry1C protein provided complete control of susceptible, $Cry1A^R$

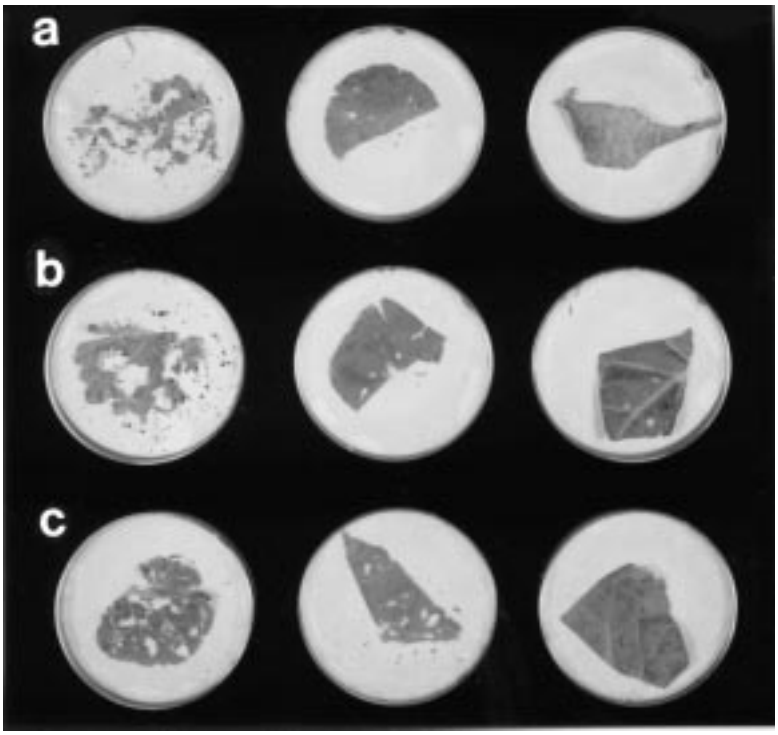

Figure 5. Insect bioassay of leaf sections of independent transgenic broccoli plants. Five third instar larvae of susceptible, Cry1A^R, or Cry1C^R *P. xylostella* were applied to leaf sections and allowed to feed for four days. Row a: susceptible larvae on leaf sections: row b: Cry1A^R larvae on leaf sections; row c: Cry1C^R larvae on leaf sections. Left in each row: leaf sections from an untransformed control plant; viable larvae are visible. Middle and right in each row: leaf sections from transgenic plants expressing very low or high levels of Cry1C protein, respectively. Dead larvae are visible on the leaf sections producing a high level of Cry1C protein.

and Cry1C^R DBM larvae at all larval developmental stages. Plants with moderate or low production of Cry1C protein showed high toxicity to larvae of susceptible and Cry1A^R DBM; however, they suffered minor to moderate defoliation and were unable to cause complete mortality of DBM larvae selected for resistance to Cry1C protein. A transgenic plant with very low expression showed a clear differential between its ability to control DBM resistant or susceptible to Cry1C protein.

The evolution of insect resistance is a major concern in agricultural use of Bt-transgenic crops, and appropriate resistance management strategies are needed to ensure its durability. To design effective resistance management programs for Bt resistance, it is important to understand the patterns of cross-resistance between different toxins that might be used in different varieties or pyramided in the same variety. High or moderate resistance to Cry1A or Cry1C proteins, respectively, have evolved in field populations of DBM. Using transgenic plants for the first time, our studies confirm earlier work [32, 34] showing that there is no cross-resistance between Cry1A and Cry1C in the DBM. Furthermore, transgenic broccoli plants expressing a range of Cry1C protein levels will allow researchers to select for Cry1C^R DBM in controlled settings to study modes and dynamics of the evolution of Cry1C resistance.

Development of Bt crops has led to vigorous debate on strategies for delaying or preventing the development of resistant insect populations [4, 11, 24]. One attractive concept is the 'high-dose/refuge' strategy. This approach aims to slow evolution of resistance by preventing the accumulation of resistant homozygotes. In this approach, it is assumed that only larvae homozygous for resistance can survive on the transgenic crop, i.e., the dose of toxin in the plant is such that resistance is expressed as a recessive trait. In the event that resistant insects emerge from the transgenic crop, the strategy also assumes that there are sufficient numbers of susceptible insects emerging from the refuge crop to minimize the frequency of resistant insects mating with each other. Since resistance is functionally recessive, heterozygotes on the transgenic crop

Figure 6. Whole plant insect bioassay of transgenic broccoli. Intact plants were assayed for control of susceptible late second instar DBM larvae. Larvae were allowed to feed on each plant for five days. Left: untransformed control; viable larvae are visible. Right: transgenic plant with high expression of the *cry1C* gene.

will be killed, and R alleles will be removed from the insect population [20–24].

Thus far, our experimental results support using the high dose/refuge strategy for delaying resistance to both Cry1A and Cry1C in DBM. We have previously shown that heterozygous Cry1A^R larvae are killed by Cry1A transgenic broccoli plants, indicating that the dose of toxin in the plants is high enough to cause Cry1A resistance in the larvae to be expressed as a recessive trait [13]. In this paper, we show that transgenic broccoli expressing high levels of Cry1C protein not only completely eliminated susceptible and Cry1A^R DBM larvae but also caused 100% mortality of DBM larvae from a population that could be considered moderately resistant to Cry1C, i.e. about 100-fold more resistant than our susceptible colony. Presumably, a moderately resistant population has a significant proportion of larvae that are heterozygous for resistance, suggesting that the dose of Cry1C toxin in our high-expressing plants is also high enough to render Cry1C resistance recessive.

The high-dose/refuge strategy may not be appropriate if resistance is not entirely recessive. In such cases, reducing selection by using plants that express toxin at a lower dose together with biological and cultural methods of control may be the best alternative for delaying resistance [9].

Although it has been reported that one DBM gene confers resistance to four closely related Bt toxins [31], resistance appears to be due to a dramatic decrease in the toxicity of Cry1A(a), Cry1A(b), and Cry1A(c) with little cross-resistance to other toxin families such as Cry1B, Cry1C, and Cry1D [31, 34]. Our experimental results provide evidence that Cry1C-expressing transgenic broccoli plants cause complete mortality of DBM that have developed high resistance to Cry1A(c) protein, suggesting that pyramiding of resistance genes may be effective in Bt resistance management. The combination of two or more insecticidal proteins with different modes of action not only increases the insecticidal spectrum but also could delay evolution of insect resistance [20–23]. We are now combining *cry1A* and *cry1C* genes in the same broccoli plants to see whether they can confer toxicity to high infestations of both Cry1A^R and Cry1C^R DBM. Combination of Bt genes with other unrelated Bt (or

non-Bt) insect control genes to improve insecticidal activity and longevity of transgenic crops will also be tested.

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