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Development of transgenic collards (*Brassica oleracea* L., var. *acephala*) expressing a *cry1Ac* or *cry1C* Bt gene for control of the diamondback moth

J. Cao[a], A.M. Shelton[b], E.D. Earle[a,*]

[a]*Department of Plant Breeding & Genetics, Cornell University, 240 Emerson Hall, Ithaca, NY 14853, USA*
[b]*Department of Entomology, Cornell University, New York State Agricultural Experiment Station, 630 W. North St., Geneva, NY 14456, USA*

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Abstract

Collards (*Brassica oleracea* var. *acephala*) are an important vegetable crucifer produced worldwide for human consumption, and one subject to severe injury by Lepidoptera. We have produced *Bacillus thuringiensis* (Bt)-transgenic collard lines that have the potential to be used either for direct control or as a "dead end" trap crop for Lepidoptera. To produce collard lines expressing Bt genes, a *cry1Ac* Bt gene, in association with the *nptII* gene for kanamycin-resistance, and a *cry1C* Bt gene, in association with the *hpt* gene for hygromycin-resistance, were introduced into seedling explants by *Agrobacterium tumefaciens*-mediated transformation. A total of 30 kanamycin-resistant and 28 hygromycin-resistant plants were regenerated from two collard cultivars ("McCormack's Green Glaze" [glossy leaves] and "Champion" [non-glossy leaves]). PCR assays of the kanamycin- and hygromycin-resistant plants and Southern analyses of the hygromycin-resistant Champion plants confirmed the presence and integration of the associated Bt genes. ELISA analyses of leaf tissue from the antibiotic-resistant plants showed that production of the Cry1Ac and Cry1C Bt proteins varied greatly among independent transformants, ranging from undetectable to over 2000 ng of Bt protein/mg of total soluble protein. All *cry1Ac* and *cry1C* collard plants in which Bt protein was detected caused complete mortality of larvae from susceptible diamondback moth, *Plutella xylostella*. *Cry1Ac*-transgenic plants also controlled *P. xylostella* populations resistant to Cry1C protein, while *cry1C*-transgenic plants controlled Cry1Ac-resistant *P. xylostella*. Sexual crosses have produced lines with both Bt genes. "McCormack's Green Glaze" plants were more attractive for *P. xylostella* oviposition than cabbage plants and hence are suitable for use as "dead end" trap crops. If approved for human consumption in the future, Bt collards could serve not only as a trap crop but also as a cash crop, thereby expanding the traditional role of a trap crop. This example of Bt collards illustrates the potential for using biotechnology in multiple ways for insect control.
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1. Introduction

Bacillus thuringiensis (Bt) genes have been introduced into several crops for protection against insect pests. Worldwide in 2003 more than 18 million ha of Bt crops, primarily corn and cotton, were grown (James, 2003). More than 11 million hectares (28 million acres) of Bt-transgenic insecticidal crops (corn and cotton) were planted in the US in 2003 (USDA, 2003). Effective insect control has also been achieved in Bt-transgenic crucifer vegetables (Earle et al., 2004) but no such products have yet been released commercially because of economic, environmental and social concerns. Such concerns may eventually be resolved, allowing the use of Bt crucifers for direct control of insect pests. An

*Corresponding author. Department of Plant Breeding, Cornell University, 240 Emerson Hall, Ithaca, NY 14853-1901, USA. Tel.: +1 6072553102; fax: +1 6072556683.
E-mail address: ede3@cornell.edu (E.D. Earle).

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alternate use of Bt technology for crop protection is the creation of a Bt-transgenic trap crop, an approach that may provide a helpful complement to current integrated pest management (IPM) strategies.

Trap crops that are highly attractive to insect oviposition may reduce insect pressure on the cash crop (Hokkanen, 1991). It has been reported that collards (*Brassica oleracea* L. var. *acephala*) and Indian mustard (*Brassica juncea* L.) are preferred over cabbage for oviposition by *Plutella xylostella* L. and that they harbor a higher number of larvae (Srinivasan and Moorthy, 1991). They have been evaluated as trap crops to protect cabbage from *P. xylostella* in Asia, Africa, and the United States (Srinivasan and Moorthy, 1992; Luther et al., 1996; Charleston and Kfir, 2000; Mitchell et al., 2000), with varied results. However, neither collards nor Indian mustard are toxic to the larvae that hatch after oviposition. Therefore, insecticide sprays were still needed, although in reduced frequency, to prevent the spread of the trapped insects back to the cabbage.

"Dead-end" trap crops, on which the larvae do not survive, are an even more promising concept (Shelton and Nault, 2004). The toxicity to insects could either be an intrinsic feature of the trap crop as in *Barbarea vulgaris* (Idris and Grafius, 1996; Shinoda et al., 2002; Shelton and Nault, 2004) or introduced into it. We are interested in the concept that collards and Indian mustard could be more effective trap crops if they killed lepidopteran insects by virtue of expression of a suitable insecticidal transgene. Collards are particularly attractive for this purpose for several reasons. Because of their growth habits over the course of the season, they provide very large leaf area for oviposition. Moreover their biennial habit means that they will not set seed during the growing season. The plants could readily be destroyed before flowering, thus avoiding problems related to possible gene flow via pollen.

Efficacy of proteins from Bt in control of lepidopteran insects is well documented (Koziel et al., 1993; Perlak et al., 1990). Previous studies in our and other laboratories have showed excellent control of *P. xylostella* by *B. oleracea* plants carrying a synthetic or fully modified *cry1* Bt gene (Metz et al., 1995; Cao et al., 1999; Jin et al., 2000; Bhattacharya et al., 2002). Furthermore, broccoli plants expressing both *cry1Ac* and *cry1C* genes caused rapid and complete mortality of *P. xylostella* larvae resistant to Cry1A or Cry1C protein (Cao et al., 2002).

Transgenic plants of diverse *Brassica* species have been obtained by *Agrobacterium tumefaciens*-mediated transformation of seedling or flower stalk explants (Fry et al., 1987; Kuvshinov et al., 2001). We have successfully introduced a number of Bt genes into broccoli, Chinese cabbage, cabbage, and cauliflower (Metz et al., 1995; Cao et al., 1999; Cho et al., 2001; Cao et al., 2003). To date, however, there are no reports of production of transgenic collards.

In this paper, we report the regeneration of two types of collards ("Champion" and "McCormack's Green Glaze") carrying either a *cry1Ac* or a *cry1C* gene and demonstrate the production of Cry1Ac or Cry1C protein as well as effective control of susceptible and resistant lines of *P. xylostella*. Our results suggest that introduction of Bt genes into collards could be useful both for direct control of *P. xylostella* and also as part of a trap crop system for protection of a non-transgenic crucifer crop such as cabbage. The materials produced will permit tests of whether Bt collards are a more effective trap crop than non-transgenic collards in protecting cabbage plants from insect attack and yield loss. Success in implementation of this approach might also encourage more future exploration of biotechnology combined with conventional biological control methods for sustainable agriculture.

2. Materials and methods

2.1. Cultivars

Two varieties of collard (*B. oleracea* L. var. *acephala*) were used. Seeds of "Champion", a line with non-glossy leaves, were kindly provided by Dr. Mark Farnham (USDA-ARS-US Vegetable Laboratory, Charleston, SC). Seeds of the uniformly glossy leaf line "McCormack's Green Glaze" (MGG) were purchased from Garden Medicinals (Earlysville, VA). The cytoplasmic male sterile (CMS) cabbage (*B. oleracea* L. var. *capitata*) plants used as female parents in crosses carried the "Anand" type of CMS. This CMS was transferred from rapid-cycling *B. oleracea* (Cardi and Earle, 1997) into cabbage by one or more backcrosses with cabbage line NY 4002.

2.2. Binary vectors

The binary vectors pNS_6 and pMON10517 (Cao et al., 2002) were used to transform seedling explants. The pNS_6 vector carried a CaMV *35S 5′/cry1C/35S 3′* expression cassette associated with the *hpt* gene for hygromycin selection while pMON10517 carried a CmoV *35S 5′/ cry1Ac/E9 3′* expression cassette associated with the *nptII* gene for kanamycin selection.

2.3. Transformation

The *B. oleracea* transformation protocols routinely used in our laboratory (Metz et al., 1995; Cao et al., 1999) were followed for transformation of collards. Briefly, hypocotyls and cotyledonary petioles from 7–10 d old seedlings germinated in vitro were used for

A. tumefaciens-mediated transformation. Transgenic shoots carrying the *cry1Ac* or *cry1C* gene were selected on Murashige–Skoog (MS) (1962) regeneration medium containing kanamycin (25 mg/L) or hygromycin (10 mg/L), respectively.

2.4. DNA isolation, PCR and Southern blot analyses

Genomic DNA was isolated from leaf tissues as previously described (Cao et al., 1999). PCR analysis of genomic DNA from in vitro plantlets was carried out as described in Cao et al. (2003) using the following primers: *cry1Ac* gene: 5′ primer 5′-CAACTAGGTCAGGGTGTC-3′; 3′ primer: 5′-AGCGCATCTGTTAGGCTC-3′; *cry1C* gene: 5′ primer, 5′-GGAGAAAGATGGGGATTG-3′; 3′ primer, 5′-AACTCGTGCATCCCTACT-3′. For Southern blot analysis, genomic DNA was digested with an appropriate restriction enzyme, electrophoresed on a 1% agarose gel, and blotted onto nylon membrane. Blots were probed with a non-radioactive DIG-labeled *Bam*H I-cleaved 1.9 kb DNA fragment covering the entire coding region of the *cry1C* gene. Random primed DNA labeling and subsequent detection of hybridization products were performed following the manufacturer's instructions with a digoxigenin-dUTP DNA labeling and detection starter kit II from Roche Molecular Biochemicals (Cat. No. 1 585 61).

2.5. ELISA assays

Total soluble proteins were isolated from leaf tissues of *cry1Ac* or *cry1C* plants or controls as previously described (Cao et al., 2001). Soluble protein concentrations were determined according to the BioRad protein assay (Catalog No. 5000-0006). Protein dilution and ELISA were performed according to the manufacturer's instructions with the Cry1C or Cry1Ab/Cry1Ac plate kits from Envirologix (Portland, ME). The O.D. values of the Bt proteins were determined by a microplate reader set at 450 nm. The amount of Cry1C or Cry1Ac protein was calculated from standard concentrations obtained with Cry1C or Cry1Ac (Envirologix) and expressed as ng Bt protein/mg total soluble protein.

2.6. Insect bioassays

Larvae of susceptible, Cry1A-resistant ($Cry1A^R$), and Cry1C-resistant ($Cry1C^R$) *P. xylostella* strains were used for insect bioassays. At the time of the bioassays, the $Cry1A^R$ population was over 280,000-fold resistant to Cry1Ac protoxin (J.-Z. Zhao, unpublished data) but was susceptible to Cry1C-expressing broccoli (Cao et al., 1999). The $Cry1C^R$ strain was over 500-fold resistant to Cry1C protoxin but was susceptible to Cry1Ac-expressing broccoli (Zhao et al., 2001). The *P. xylostella* colonies were kept in an environmental chamber at 27 °C ± 1 °C, 35% ± 2% RH, and photoperiod of 16/8 (light/dark). Prior to bioassays, eggs were hatched and larvae reared on artificial diet (Shelton et al., 1991).

Detached leaf bioassays were performed using 2nd instar larvae of susceptible, $Cry1A^R$, and $Cry1C^R$ *P. xylostella* strains. A leaf from *cry1Ac* or *cry1C* plants or untransformed collard plants grown either in soil or in vitro was placed in a baby food jar containing 1% Phytagar. Five susceptible, $Cry1A^R$, or $Cry1C^R$ larvae were placed on the surface of the leaf and maintained at 25 °C under a 16/8 h light/dark regime. All insect bioassays were performed in triplicate. Leaf damage (estimated visually) and insect mortality were scored after 4 d. Mortality is presented as simple numerals because there was no variation among replicates. Similar detached leaf assays were conducted with 2nd instar larvae of cabbage looper (*Trichoplusia ni*), another Lepidopteran pest of *B. oleracea*. For assays with whole plants, each of 4–6 leaves of a plant was infested with five 2nd instar larvae of susceptible *P. xylostella*. Each plant was placed inside a plastic bag and maintained at 25 °C under a 16/8 h light/dark regime. Leaf damage and insect mortality were evaluated after 5 d.

2.7. Tests of ovipositional preference

Ovipositional tests were done using a two-choice method comparing each plant species to cabbage (*B. oleracea*, var. *capitata*), variety 'Huron', using three replicates of each choice test. A single leaf of each plant type was placed into a 50 mL flask filled with water, and the lip of the flask was sealed with Parafilm. A flask of one plant type was placed into a 1 m^3 chamber along with a flask of a cabbage leaf. Care was taken to use only leaves of a similar size. Newly emerged *P. xylostella* adults (3 female and 3 male) were introduced into each chamber. Moths were allowed to mate and lay eggs for 24 h after which the eggs were counted. Each leaf was placed with its flask into a smaller chamber for 7 d at which time the number of surviving larvae was counted.

2.8. Recovery of progeny of transgenic collard plants

Plants with 8–10 leaves growing in soil were vernalized for 10 weeks in a growth chamber maintained at 4 °C. The temperature was then gradually raised to 20 °C over a period of 5 d. After one week at room temperature, plants were transferred to a greenhouse for seed production. Flowers were bud-pollinated with pollen collected from open flowers of the same plant. Inheritance and segregation of Bt transgenes in the seeds recovered were determined using a leaf-based callus induction method (Cao et al., 2002). Briefly, seeds were germinated on MS medium with no plant growth regulators. Pieces of the first true leaf were placed on MS medium containing BA (5 mg/L) and NAA (0.5 mg/

L) as well as hygromycin (20 mg/L) as a selection agent. Transgenic explants showed callus formation and shoot regeneration while control and non-transgenic ones were bleached. Clonal progeny were obtained in vitro as described in Cao and Earle (2003).

3. Results

3.1. Recovery of transgenic collard plants

A synthetic *cry1C* Bt gene associated with the *hpt* gene or a *cry1Ac* Bt gene associated with the *nptII* gene was introduced into glossy leaf and non-glossy collards through *Agrobacterium*-mediated transformation of seedling explants. Hygromycin or kanamycin was initially used to screen for transgenic plants after co-cultivation of explants with *Agrobacterium* cells carrying the Bt gene. On hygromycin selection medium, most explants stopped growth and development and turned brown within the first two subcultures. Some shoots that formed on explants remained green and gave rise to additional green shoots when continuously cultured on regeneration medium containing hygromycin. Explants co-cultivated with *Agrobacteria* harboring the *nptII* gene for kanamycin selection showed different patterns of development on medium containing kanamycin. During the first two subcultures many white calli and purple shoots were produced, along with some green shoots. Eventually, the purple shoots wilted, and the white calli and explants turned brown and died. Many of the green shoots also turned white and died after extended culture on medium containing kanamycin. Only about $\frac{1}{3}$ of the initially green shoots retained their green color and produced additional green shoots on medium with kanamycin. All shoots that remained green and had several green leaflets on selective medium developed normal healthy roots when transferred to rooting medium containing the selective antibiotic.

From various transformation experiments with petiole and hypocotyl explants, a total of 12 hygromycin-resistant or 24 kanamycin-resistant MGG plants were obtained from 600 and 2367 MGG explants while a total of 16 hygromycin-resistant or 6 kanamycin-resistant Champion plants were produced from 652 and 373 Champion explants respectively. The transformation efficiency (number of independent transgenic plants/explants used) ranged from 0.8% to 3.6%.

3.2. PCR analysis of the transformants

PCR analysis was carried out for rapid detection of the *cry1C* or *cry1Ac* gene in the hygromycin- or kanamycin-resistant collard plants. The expected 1.1 kb band representing the *cry1C* fragment was amplified in samples from the hygromycin-resistant plants and from the pNS6 plasmid (Fig. 1). Similarly, the expected 1.3 kb band representing the *cry1Ac* fragment was seen in samples from the kanamycin-resistant plants and the pMON10517 plasmid. Neither the 1.1 kb *cry1C* band nor the 1.3 kb *cry1Ac* band was seen in non-transformed control samples. These results presented initial evidence that the *cry1C* or *cry1Ac* gene was present in all of the tested hygromycin- or kanamycin-resistant plants, respectively.

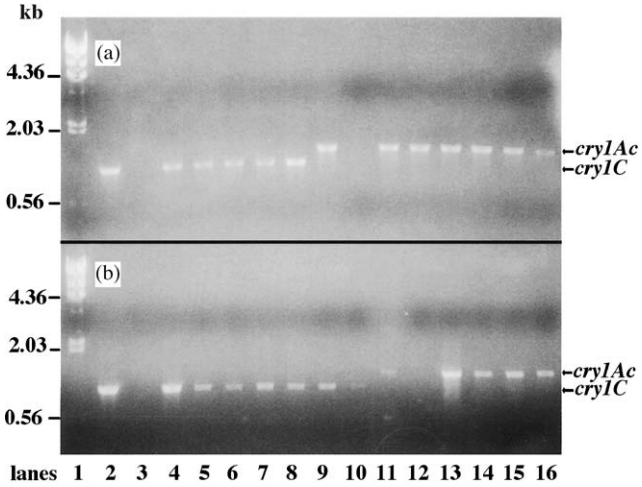

Fig. 1. PCR analysis of *cry1C* or *cry1Ac* collard plants. A, PCR analysis of genomic DNA from non-glossy Champion. PCR products in lanes 2–8 were obtained with the *cry1C*-specific primers and lanes 9–16 with *cry1Ac*-specific primers. Lane 1, *Hind* III digested lambda DNA; lane 2, pNS_6 carrying the *cry1C* gene; lane 9, pMON10517 carrying the *cry1Ac* gene; lanes 3 and 10, DNA from non-transformed Champion; lanes 4–8 from hygromycin-resistant plants; lanes 11–16 from kanamycin-resistant plants. B, PCR analysis of genomic DNA from glossy MGG. PCR products in lanes 2–10 were obtained with *cry1C*-specific primers and lanes 11–16 with *cry1Ac*-specific primers. Lane 1, *Hind* III digested lambda DNA; lane 2, pNS_6 carrying the *cry1C* gene; lane 11, pMON10517 carrying the *cry1Ac* gene; lanes 3 and 12, DNA from non-transformed MGG; lanes 4–10 from hygromycin-resistant plants; lanes 13–16 from kanamycin-resistant plants.

3.3. Southern blot analyses

Southern blot analysis was carried out with DNA samples isolated from independent *cry1C* plants. To confirm the presence and integration of the *cry1C* gene into the genome of the transformants, DNA samples were digested with *Bam*H I, *Hind* III, or *Sal* I, which release the 1.9 kb *cry1C* gene, the 3.6 kb CaMV *35S*/*cry1C* expression cassette, or various size fragments containing both introduced and host DNA, respectively. As shown in Fig. 2, no bands were detected in non-transformed plants, whereas bands representing the 1.9 kb *cry1C* gene or the 3.6 kb *cry1C* expression cassette were observed in the transgenic plants and pNS_6 plasmid DNA. Genomic DNA isolated from *cry1C* plants, digested with *Sal* I and probed with the *cry1C*

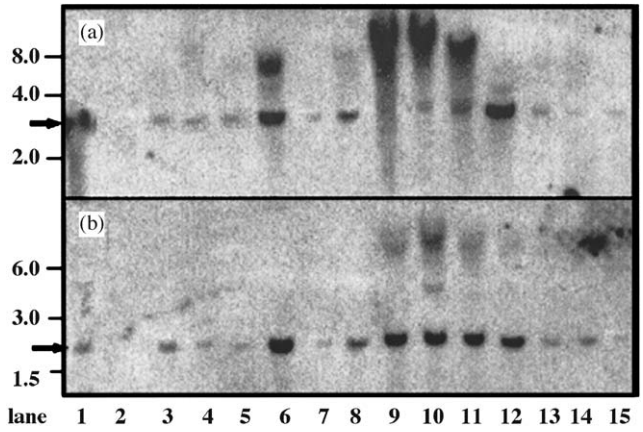

Fig. 2. Southern blot analysis of genomic DNA from independent *cry1C* Champion plants. A, DNA digested with *Hind* III, which released a 3.6 kb DNA fragment covering the whole *35S 5′/cry1C* expression cassette and B, with *Bam*H I, which released a 1.9 kb DNA fragment covering the entire *cry1C* gene. A non-radioactive DIG-labeled 1.9 kb *Bam*H I fragment from pNS_6 covering entire *cry1C* gene was used as the probe. Lane 1, pNS_6 DNA carrying *cry1C*; lane 2, non-transgenic Champion plant; lanes 3–15, independent *cry1C* Champion plants.

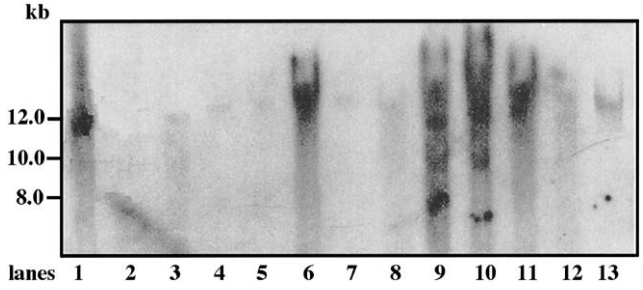

Fig. 3. Southern blot analysis showing integration of the *cry1C* gene in independent *cry1C* Champion plants. Genomic DNA isolated from independent *cry1C* plants was digested with *Sal* I, which released a DNA fragment containing 10 kb T-DNA plus an unknown size genomic DNA, and probed with the 1.9 kb *cry1C* gene. Lane 1, linearized plasmid DNA with the *cry1C* gene; lane 2, non-transgenic Champion plant; lanes 3–13 independent *cry1C* plants.

gene, exhibited bands of various sizes, all of which were higher than the 10 kb T-DNA (Fig. 3). This indicated that the higher molecular weight fragments contained both T-DNA and a portion of plant genomic DNA. The analysis also showed multiple bands in some of *cry1C* collard plants, suggesting that multiple copies of the T-DNA were inserted into their genomes. Taken together, the Southern analyses confirmed that the *cry1C* gene was integrated into the Champion genome.

3.4. ELISA assays

ELISA analysis showed that the amount of the Cry1Ac and Cry1C protein produced varied greatly among independent transformants, ranging from undetectable (<25 ng/mg total soluble protein) to over 0.2% Cry1C or 0.1% Cry1Ac protein of total soluble proteins (Table 1). All of the 16 *cry1C* Champion plants produced some Cry1C protein. Seven produced high levels (>1000 ng per mg total soluble proteins), while the rest showed moderate or low production. In contrast, only three of the 10 *cry1C* MGG plants produced detectable Cry1C protein. Four of the six *cry1Ac* Champion plants had low to moderate levels of Cry1Ac protein. Two of the 10 *cry1Ac* MGG plants assayed produced a high level of Cry1Ac protein, while five others had moderate or low levels.

Table 1
Production of Cry1C or Cry1Ac Bt protein in transgenic collard plants

Cultivar	Protein	No. of plants	Protein level	Amount (ng/mg TSP[a])
Champion (non-glossy)	Cry1C	4	Low	107–269
		5	Moderate	413–654
		7	High	1011–2036
	Cry1Ac	2	ND[b]	—
		3	Low	75–104
		1	Moderate	509
McCormack's Green Glaze (glossy)	Cry1C	7	ND[b]	—
		1	Moderate	654
		2	High	1582; 2045
	Cry1Ac	3	ND[b]	—
		4	Low	60–365
		1	Moderate	721
		2	High	1002; 1109

[a]Total soluble protein.
[b]Not detected (<ca. 25 ng).

3.5. Insect bioassays

Detached leaf bioassays were used to assay *cry1C* or *cry1Ac* collard plants for resistance to 2nd instar larvae of susceptible, $Cry1A^R$ and $Cry1C^R$ *P. xylostella* (Tables 2, 3). In these assays, control Champion and MGG leaves showed severe leaf damage (over 80% defoliation) by all three types of larvae, with 0–20% larval mortality. There was no apparent difference between the two cultivars in insect survival on control leaves detached from young plants grown either in vitro or in soil. The transgenic plants that produced no detectable Cry1Ac or Cry1C protein also suffered severe leaf damage by all three types of *P. xylostella* (Tables 2, 3), similar to the non-transgenic controls, and allowed the larvae to advance to the 4th instar stage.

In contrast, in all replicates of the bioassays, all Champion and MGG plants with moderate or high levels of Cry1C protein caused 100% mortality of susceptible and $Cry1A^R$ larvae with very little defoliation (Table 2). Champion plants with a low level of

Table 2
Control of second instar larvae of susceptible, Cry1A^R, and Cry1C^R diamondback moth by *cry1C* collard plants in detached leaf assays

Cultivar	No. of lines	Cry1C level	Susceptible larvae		Cry1A^R larvae		Cry1C^R larvae
			Defoliation (%±S.D.)	Mortality (%)	Defoliation (%±S.D.)	Mortality (%)	Defoliation (%±S.D.)
Champion	Control[a]	None	87±12	0–20	90±10	0–20	80±0
	3	Low	12±12	100	7±7	100	87±12
	4	Moderate	2±3	100	2±3	100	85±9
	6	High	1±1	100	0±0	100	87±13
McCormack's Green Glaze	Control[a]	None	80±5	0–20	82±6	0–20	87±10
	7	None[b]	83±6	0–20	75±9	0–20	88±8
	1	Moderate	1±1	100	1±0	100	77±3
	2	High	0±0	100	0±0	100	85±9

[a]Non-transgenic.
[b]*cry1C* lines with no detectable Cry1C protein.

Table 3
Control of second instar larvae of susceptible, Cry1C^R, and Cry1A^R diamondback moth by *cry1Ac* collard plants in detached leaf assays

Cultivar	No. of lines	Cry1A Level	Susceptible larvae		Cry1C^R larvae		Cry1A^R larvae
			Defoliation (%±S.D.)	Mortality (%)	Defoliation (%±S.D.)	Mortality (%)	Defoliation (%±S.D.)
Champion	Control[a]	None	90±10	0–20	87±12	0–20	90±10
	2	None[b]	90±9	0–20	85±9	0–20	83±12
	3	Low	12±12	100	12±12	100	83±15
	1	Moderate	2±3	100	2±1	100	85±9
McCormack's Green Glaze	Control[a]	None	87±6	0–20	82±6	0–20	82±6
	3	None[b]	85±10	0–20	77±8	0–20	85±9
	2	Moderate	3±1	100	4±1	100	80±9
	3	High	1±1	100	0±0	100	82±3

[a]Non-transgenic.
[b]*cry1Ac* lines with no detectable Cry1Ac protein.

Cry1C protein suffered an average of 12% defoliation with up to 25% observed in a few replicates. Larval growth was severely inhibited on all of these plants, and all larvae died by 4 d after infestation. None of the *cry1C* plants controlled Cry1C^R *P. xylostella* larvae (Table 2).

Similarly, in all replicates, the *cry1Ac* Champion plant with a moderate level of Cry1Ac protein effectively controlled both susceptible and Cry1C^R *P. xylostella* larvae with very little defoliation (Table 3). Champion plants with a low level of Cry1Ac protein also caused complete mortality but suffered slightly more leaf damage. The MGG plants with moderate or high levels of Cry1Ac protein controlled susceptible and Cry1C^R resistant larvae with 100% mortality and little or no defoliation (Table 3). The *cry1Ac* plants did not control the Cry1A^R larvae (Table 3).

To evaluate the control of *P. xylostella* in intact Bt collard plants, a glossy and a non-glossy plant with high production of Cry1C protein and severe toxicity to *P. xylostella* in the detached leaf assay were infested with five 2nd instar susceptible *P. xylostella* larvae per leaf. After 5 d, non-transformed control plants were severely defoliated. Larvae placed on them had advanced to the 4th instar, and many had pupated. In contrast, the high expressing *cry1C* plants showed little or no feeding damage and caused 100% mortality within 2 d after the infestation.

The Cry1C-producing collard plants also showed strong insecticidal activity against larvae of *T. ni* in detached leaf assays (Table 4). They caused complete mortality of *T. ni* larvae within 4 d with little or no defoliation, in contrast to the highly susceptible non-transgenic controls.

3.6. Ovipositional preference on glossy and non-glossy plants

Studies of ovipositional preference showed that *P. xylostella* strongly preferred to lay eggs on the glossy MGG leaves rather than on cabbage (Table 5). There was no significant difference in oviposition on leaves of non-glossy Champion collards and the cabbage check.

Table 4
Control of second instar larvae of *Trichoplusia ni* by Bt collard plants in detached leaf assays

Cultivar	No. of lines	Bt gene	Expression of Bt gene	Defoliation (%±S.D.)	Mortality (%)
Champion	Control[a]	None	None	92±8	0–20
	4	*cry1C*	Moderate	5±5	100
	6		High	1±2	100
McCormack's Green Glaze	Control[a]	None	None	90±10	0–20
	2	*cry1C*	High	1±1	100
	2	*cry1Ac*	Moderate	4±2	100
	5		High	0±1	100

[a]Non-transgenic.

The presence of the *cry1C* gene in the collard lines did not alter ovipositional preference. None of the *P. xylostella* larvae that hatched from the eggs laid on either *cry1C*-transgenic line survived, but larval survival was lower on the control MGG leaves than on control Champion leaves.

3.7. Recovery of progeny from Bt collard plants

Sexual progeny were obtained from *cry1C* Champion and MGG plants or *cry1Ac* Champion plants through selfing via bud pollination. Segregation analysis of progeny from two MGG plants with high expression of *cry1C* showed that both had a single integration of T-DNA carrying the *cry1C* gene into their genome. We also successfully crossed *cry1Ac* Champion with *cry1C* MGG plants in order to obtain progeny carrying both Bt genes. Furthermore, CMS *cry1C* or *cry1Ac* F_1 seeds were produced by pollinating CMS cabbage with pollen from *cry1C* or *cry1Ac* Champion plants or *cry1C* MGG plants. Recovery of selfed progeny from *cry1Ac* MGG plants as well as production of *cry1Ac*+*cry1C* MGG lines and CMS MGG-cabbage hybrids are in process. Clonal progeny were readily obtained via the procedure of Cao and Earle (2003). Over 500 clonal rooted plantlets ready for transfer into soil were recovered from a single *cry1C* MGG plant within four months.

Table 5
Ovipositional preference and larval survivorship of *Plutella xylostella* in glossy (McCormack's Green Glaze) and non-glossy (Champion) collards and in cabbage

Plant material	*cry1C* Bt gene	Ovipositional ratio[a]	Larval survivorship[b] (%)
Collard			
Glossy	No	7.9a	31b
	Yes	7.6a	0c
Non-glossy	No	1.3b	67a
	Yes	1.2b	0c
Cabbage (check)	No	1.1b	65a

Means within a column followed by different letters are significantly different, $P \leqslant 0.05$ (Fisher's Protected LSD, SAS Institute, 1999).

[a]Number of eggs laid on the leaf of the plant, compared to the number of eggs laid on the cabbage leaf in the same chamber for each replicate.

[b]Percent of *P. xylostella* larvae that survived from the eggs laid on each leaf.

4. Discussion

Collards are important leafy green vegetables produced worldwide and grown throughout the year in the southeastern US. To our knowledge, there are no previous reports on transformation of collards. The Bt collards described here show potential to be used either as a direct control of *P. xylostella* and other susceptible Lepidoptera or as a potential dead end trap crop for such Lepidoptera. The procedures used provide a simple method for *A. tumefaciens*-mediated transformation with either hygromycin or kanamycin selection. Plants selected for resistance to hygromycin or kanamycin contained the associated Bt genes as well. The transformation procedure was successful with both glossy and non-glossy collard plants. The plants recovered had normal phenotypes both in the vegetative and flowering stages.

As seen in our previous work with other *Brassica* materials and studies from other laboratories (Cao et al., 1999, 2002; Stewart et al., 1996), production of Bt proteins varied greatly among independently transformed *Brassica* plants. Production of Cry1C or Cry1Ac protein in collards was comparable to that in Bt broccoli and Bt cauliflower (Cao et al., 1999, 2003), ranging from undetectable to over 0.2% of total soluble proteins.

All collard plants producing detectable levels of Bt proteins caused 100% mortality of 2nd instar *P. xylostella* within 4–5 d; however, the plants with the lower levels of Bt protein suffered a little more leaf damage. As expected, the plants with no detectable

production of Bt proteins suffered severe defoliation and showed no control of *P. xylostella*.

It has been reported that larval survival and mining damage by *P. xylostella* are significantly lower on glossy-leaf *B. oleracea* genotypes than non-glossy ones (Eigenbrode et al., 1991b). Neonate *P. xylostella* larvae moved more rapidly, spent more time walking, and engaged in searching behaviors more often on glossy leaves than regular ones (Eigenbrode et al., 1991a). These characteristics of larval behavior were attributed to distinct leaf waxes that may condition resistance to *P. xylostella* in glossy types. Although non-transgenic glossy collards may provide superior control of *P. xylostella* than non-glossy types, the Bt-transgenic collards were even more effective, quickly killing all *P. xylostella* larvae. Moreover, they were equally toxic to another lepidopteran pest of crucifers (*T. ni*) and, based on previous work with the *cry1C* gene (Cao et al., 1999; Cho et al., 2001), will control imported cabbage worms (*Pieris rapae*) as well.

Oviposition preference on a trap crop is an important factor in successful management of insect populations. Recent studies have compared oviposition of *P. xylostella* on several potential trap crops for crucifer vegetables (Badenes-Perez et al., 2004). Glossy collards were much more attractive for *P. xylostella* oviposition than cabbage or non-glossy collards, which showed no significant differences. The differences in the oviposition on the two types of collards are probably due to differences in the volatiles that they produce (Badenes-Perez et al., 2004). Our studies show that *P. xylostella* ovipositional preference for glossy collards is not affected by the addition of the *cry1C* Bt gene, consistent with earlier work showing that *P. xylostella* does not distinguish between *cry1Ac* and non-transgenic broccoli (Tang et al., 1999). Since diamondback moth preferred glossy plants for oviposition and thus, efficacy was stronger for glossy lines, we will focus on them for further work. This will include production of glossy lines with uniformly high expression of both *cry1Ac* and *cry1C* genes to expand the spectrum of *P. xylostella* control, as has been achieved in broccoli (Cao et al., 2002), and to retard the evolution of resistant insect populations (Zhao et al., 2003).

Use of "dead end" trap plants for protection of cash crops is a promising concept, and Bt collards have some advantages over some of the other proposed trap crops such as *B. vulgaris*. First, the larger leaf surface area of Bt collards may prove more attractive to ovipositing Lepidoptera than *B. vulgaris*. Furthermore, *B. vulgaris*, while resistant to *P. xylostella*, is susceptible to some other important cruciferous Lepidoptera (e.g., *P. rapae*) and is not commonly used as a food crop. On the other hand, collards are an important food crop but one that is subject to severe insect attack, especially by *P. xylostella* in southern regions of the US. Consequently, it is heavily treated with insecticides. If Bt collards were approved by the US Environmental Protection Agency (EPA), they might serve to produce a marketable crop and reduce the use of more hazardous insecticides. Bt collards also have the potential advantage of not only being used as an expendable trap crop for other non-collard cruciferous vegetables (e.g. cabbage) during the season but being a cash crop that could be consumed at the end of the season. However, those who may choose not to market or consume the Bt collard trap crop would have benefited from its use in preventing insect injury and insecticide use on the non-Bt cash crop. The fact that the glossy collards are easily distinguishable from both cabbage and non-glossy collards reduces the possibility of confusion between the transgenic and non-transgenic plants.

The collard system we have described also lends itself to further potential uses. Despite the fact that no insects have developed resistance to Bt plants in the field (Tabashnik et al., 2003), there is a continuing concern about resistance since some insect species have shown the ability to develop resistance to Bt proteins (Shelton et al., 2002). A fundamental part of the EPA-mandated insecticide resistance management program for Bt plants is to have an adjacent or nearby refuge planting in which Bt susceptible alleles can be maintained within the insect population. In the Bt crucifer system we have described, use of non-Bt glossy collards that are highly attractive for oviposition but on which Lepidoptera may survive could serve as an enhanced refuge for Bt collards.

Availability of glossy Bt collards will allow tests in greenhouse and field trials to determine the usefulness of Bt collards for direct insect control or use as a trap crop. Although the biennial habit of collards means that no seeds are produced in the first season, use of CMS collard lines with pyramided *cry1Ac* and *cry1C* genes could further reduce concerns about gene flow to other crucifers. Toward this end we have pollinated CMS cabbage with Bt collard pollen and have produced CMS F_1 hybrid seeds. Further backcrosses of these CMS F1 hybrids with Bt collard will produce CMS Bt collard lines. Recently, we have also introduced Bt genes into Indian mustard plants, another proposed trap crop for cabbage pests (Badenes-Perez et al., 2004). Successful combination of biotechnology with the familiar biocontrol practice of trap cropping could open a new way to reduce insect damage in cabbage and other crucifer vegetables without having a transgenic commercial crop. This approach may be relevant to other types of crops as well.

The Bt Champion plants did not show enhanced *P. xylostella* oviposition in comparison to cabbage, so they are unlikely to be useful as dead-end trap crops. They would, however, provide strong direct control of lepidopteran pests of a commercial collard crop.

5. Conclusion

We have introduced the *cry1Ac* and *cry1C* Bt genes into glossy and non-glossy collard plants. Some of these plants produced high levels of Bt proteins (>1000 ng/mg of total soluble protein) and provided excellent control of susceptible and resistance types of *P. xylostella*, as well as *T. ni*. Availability of progeny of these transgenic plants will allow field tests of their effectiveness as a direct method of insect control or as enhanced trap crops to protect cabbage against *P. xylostella* and other lepidopteran insect pests.

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