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Broccoli plants with pyramided *cry1Ac* and *cry1C* Bt genes control diamondback moths resistant to Cry1A and Cry1C proteins

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Abstract This study was undertaken to determine the effects of pyramiding two *Bacillus thuringiensis* (Bt) genes in the same plant on the production of Bt proteins and the control of diamondback moths (DBM, *Plutella xylostella*) resistant to one or the other protein. Broccoli lines carrying both *cry1Ac* and *cry1C* Bt genes were produced by sexual crosses of *cry1Ac*- and *cry1C*-transgenic plants. Plants containing both genes were selected by tests for resistance to kanamycin and hygromycin, and confirmed by PCR analysis for the Bt genes. Both *cry1Ac* and *cry1C* mRNAs were detected in the hybrid lines, and Cry1Ac and Cry1C proteins were stably produced at levels comparable to the parental plants. Plants producing both Cry1Ac and Cry1C proteins caused rapid and complete mortality of DBM larvae resistant to Cry1A or Cry1C, and suffered little or no leaf damage. These plants, in combination with the resistant DBM populations available, will allow greenhouse or field studies of resistance management strategies involving gene pyramiding.

Keywords *Bacillus thuringiensis* · *Brassica oleracea* · *Plutella xylostella* · Pyramiding · Resistance management

Introduction

Development of crops expressing genes from the bacterium *Bacillus thuringiensis* (Bt) has revolutionized our ability to control insect pests. Insect-resistant Bt-transgenic crops were first grown commercially in 1996 (Krattiger 1997). Since then, the acreage of Bt crops has increased steadily. In 1999, an estimated 26% of corn (EPA 1999a) and 32% of cotton (Carpenter and Giannessi 2000) grown in the U.S. contained a transgene for an insecticidal protein derived from Bt.

Bt-transgenic crops have considerable advantages both for the environment and for farm-worker safety, but the prevalence of Bt crops has raised concerns that these gains will be short-lived due to the possible emergence of Bt-resistant pests. Implementation of successful resistance management strategies could keep the resistance allele frequency low enough in target insect populations to prevent, or delay, problems with insect resistance to Bt proteins. Various deployment strategies have been suggested for crops containing insecticidal proteins or other traits that provide resistance to insect pests (McGaughey and Whalon 1992). These include use of non-transgenic refuges on which susceptible insects can survive, use of multiple insect control genes, and control of the level or specificity of expression. The only currently available strategy is the use of a high dose of a single gene with a refuge (US EPA 1998). In corn, for example, the US EPA required registrant companies to ensure that corn growers plant a minimum structured refuge of at least 20% non-Bt corn for the 2000 growing season. A refuge of at least 50% non-Bt corn was required in areas where Bt cotton is grown (US EPA 1999b), because the corn earworm (*Helicoverpa zea*) is a pest on both crops. Refuges allow pests to feed on plants lacking toxins, thereby maintaining Bt-susceptible alleles within the insect population. Since insect resistance to Bt is generally considered recessive (Tabashnik 1994), hybrids between Bt-susceptible and Bt-resistant insects will be controlled by the transgenic plants. In greenhouse cage studies we have demonstrated that evolution of resistant insects is delayed when a refuge is provided, with a 20% separate refuge producing the most-pronounced effects (Tang et al. 2001). However, the non-Bt cultivars will sustain yield losses from insect damage. Moreover, the refuge sizes seen as

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commercially and practically acceptable may be too small to provide a comfortable margin for the delay of resistance. Refuge size and management continues to be discussed for the re-registration of Bt crops in the U.S. (US EPA 2001). Thus there is an urgent need for new approaches to insect resistance management.

Simultaneous deployment of two or more plant insect resistance traits may be an effective way to inhibit pest adaptation, even with smaller and more economically acceptable refuge sizes. Mixtures of resistance genes can be achieved by inter-planting two cultivars, each with a different genetic basis for resistance, or by introducing two different resistance genes into the same cultivar (gene pyramiding). Computer simulations predict that gene pyramiding is much more effective in the delay of the evolution of a resistant insect population even with a smaller refuge size (Roush 1997a, b). Some experimental data on the simultaneous deployment of multiple insect resistance genes is now available. Greenplate et al. (2000) and Stewart et al. (2001) demonstrated that cotton producing two Bt gene products had greater insecticidal activity than cotton that carried only the *cry1Ac* gene. Maqbool et al. (2001) reported that rice plants carrying three insecticidal genes (*cry1Ac*, cry2A, and the snowdrop lectin gene, *gna*) showed broad resistance against a range of different rice pests. Zhao et al. (1999) evaluated transgenic tobacco expressing the *cry1Ac* Bt gene and a gene encoding a cowpea trypsin inhibitor (*CPT1*), and showed that the plants with both genes delayed the evolution of resistance in *Helicoverpa armigera* in comparison to plants carrying only the *cry1Ac* gene. Thus, crops pyramided with high doses of two or more insect resistance genes can provide promising insect control and may be useful for resistance management. However, no information is available about how well plants with multiple resistance genes control insect populations resistant to one or more of the pyramided genes.

In our previous studies, we produced transgenic broccoli plants carrying either a *cry1Ac* gene (Metz et al. 1995a, b) or a *cry1C* gene (Cao et al. 1999). Using these plants, we selected and further characterized strains of DBM populations resistant to either the Cry1A or Cry1C protein (Tang et al. 1997, 1999; Zhao et al. 2000, 2001). We showed that both Cry1A and Cry1C resistance in DBM strains is controlled by one or a few autosomal and incompletely recessive genes with little cross-resistance. With the availability and understanding of these materials, we have produced *cry1Ac* + *cry1C* hybrid broccoli lines for the study of insect resistance management. In this paper, we demonstrate that both Bt genes are stably expressed in the hybrid lines, and that the *cry1Ac* + *cry1C* hybrids cause rapid and high mortality of both Cry1A-resistant ($Cry1A^R$) and Cry1C-resistant ($Cry1C^R$) DBM.

Materials and methods

Production and selection of *cry1Ac* + *cry1C* hybrid broccoli lines

We have previously described the production and characterization of broccoli (*Brassica oleracea* L., var. '*italica*' "Green Comet") lines expressing a *cry1Ac* gene (Metz et al. 1995a) or a *cry1C* gene (Cao et al. 1999). All *cry1C* parental lines used for crossing were heterozygous with a single copy of the *cry1C* gene. Two of the *cry1C* lines (H1 and H3) produce moderate and low levels of *cry1C* mRNA and Cry1C protein, while lines H12 and H14 have high levels of *cry1C* expression and Cry1C protein (about 1.1–1.2 μg of Cry1C protein per g of fresh leaf tissue). Plants with high, moderate or low expression all effectively killed $Cry1A^R$ DBM (Cao et al. 1999). The *cry1Ac* line Q23 is a high *cry1Ac* expresser which produces about 0.6 μg of Cry1Ac protein per g of fresh leaf tissue; in contrast, *cry1Ac* line Q7 produces only marginally detectable Cry1Ac protein. Both Q23 and Q7 have a single copy of the *cry1Ac* gene. At the time of crossing, Q23 was heterozygous while Q7 was homozygous. Various cross combinations were made between these parental lines, always using a plant with the *cry1C* expression cassette (Fig. 1) as the maternal parent, and a plant with the *cry1Ac* expression cassette (Fig. 1) as the male parent. Stamens of *cry1C* flowers were removed before the buds had opened, and stigmas were immediately pollinated with *cry1Ac* pollen.

To select *cry1Ac* + *cry1C* hybrids, seeds from the crosses were germinated on MS (Murashige and Skoog 1962) medium containing hygromycin (50 mg/l). DNA was isolated from hygromycin-resistant seedlings, and PCR was carried out to identify those that contained the *cry1Ac* gene. Alternatively, a leaf piece assay was performed to select *cry1Ac*+*cry1C* hybrid plants. Seeds from the crosses were germinated on MS medium without hygromycin. Half of a young leaf was then placed on MS medium containing hygromycin (20 mg/l) while the other half was placed on MS medium containing kanamycin (50 mg/l). Plants whose leaves formed callus in the presence of both antibiotics were subjected to PCR analyses to confirm that they carried both Bt genes.

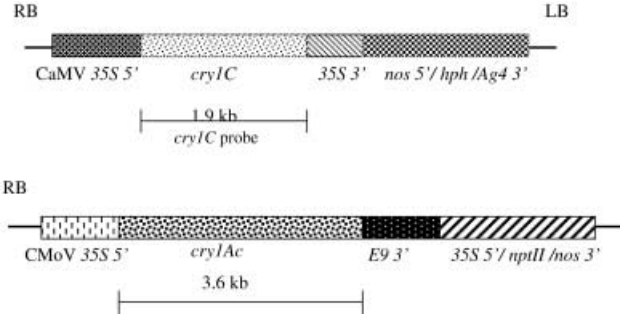

Fig. 1 Expression cassettes of the *cry1Ac* and *cry1C* genes present in the parental broccoli plants used to produce the *cry1Ac* + *cry1C* hybrids. Expression of the *cry1C* gene is regulated by the CaMV 35S promoter (*35S 5′*) and terminated by the polyadenylation sequence from the 35S RNA gene of CaMV (*35S 3′*). The 1.9-kb *Bam*HI fragment containing the entire *cry1C* gene was used as a probe for detection of *cry1C* mRNA. The hygromycin expression cassette is associated with the *cry1C* gene cassette. Expression of the *cry1Ac* gene is regulated by a caulimovirus 35S promoter (CMoV*35S 5′*) similar to the CaMV 35S promoter and terminated by the 3′ nontranslated region of the pea rbcS-E9 gene (*E9 3′*). A 1.3-kb PCR fragment containing part of the *cry1Ac* gene was used as a probe for detection of *cry1Ac* mRNA. The kanamycin expression cassette is associated with the *cry1Ac* gene expression cassette. The *cry1Ac* expression cassette carries only the right T-DNA border sequence

Analysis of hybrid lines

DNA isolation and PCR analysis

Total DNA was isolated from leaf tissues and PCR analysis was carried out as previously described (Cao et al. 1992). The PCR primers used were as follows: *cry1Ac* gene: 5′ primer: 5′-CAACTAGGTCAGGGTGTC-3′; 3′ primer: 5s′:AGCGCATCTGTTAGGCTC-3′, *cry1C* gene: 5′ primer: 5′-GGAGAAAGATGGGGATTG-3′; 3′primer: 5′-AACTCGTGCATCCCTACT-3′.

Northern-blot analysis

Total RNAs were extracted from leaf tissues as previously described (Cao et al. 1999). Northern analysis of the RNA samples was carried out following the protocol of Sambrook et al. (1989). RNAs (20 μg) were separated on agarose–formaldehyde gels and blotted onto a nylon membrane. Radioactive probes were prepared by the oligolabeling procedure (Boehringer) using α-^{32}P-dCTP, and unincorporated nucleotides were subsequently removed through mini Quick Spin DNA Columns (Boehringer). The membrane was first hybridized with a ^{32}P-labeled 1.9-kb *Bam* HI fragment containing the entire *cry1C* gene, a 1.3-kb PCR fragment containing part of the *cry1Ac* gene, and a 1.5-kb actin gene fragment from *Brassica*. After the ^{32}P-labelled probes had been stripped from the membrane, the membrane was re-hybridized with only the *cry1C* and actin probes.

Protein assays

Detection and quantitative determination of the amount of Cry1Ac and Cry1C proteins produced in *cry1Ac+cry1C* hybrid plants were monitored by enzyme-linked immunosorbant assays (ELISA) using kits from EnviroLogix Inc. (Portland, Me.). Proteins from an untransformed Green Comet broccoli plant were used as the control. Proteins were extracted from two 8-mm leaf discs and dissolved in 0.5 ml of Extraction/Dilution Buffer (EnviroLogix). ELISA was performed according to the manufacturers' instructions with Cry1Ab/Cry1Ac or Cry1C kits. Sample extracts were used at a dilution of 1:11. The o.*D*. value of samples was measured by a microplate reader set at 450 nm. The amount of Cry1Ac or Cry1C protein present was calculated from standard curves obtained with Cry1Ac or Cry1C protein (EnviroLogix). The o.*D*. value of the untransformed Green Comet control was subtracted before the determination of the concentration of Cry1Ac or Cry1C protein.

Insect bioassays

Diamondback moth strains

Larvae of Cry1A^R and Cry1C^R DBM strains were used in bioassays. The Cry1A^R strain was collected in 1994 and reared on Cry1Ac-expressing broccoli plants (Metz et al. 1995a) for more than 75 generations. Compared to the LC_{50} of the susceptible strain, the Cry1A^R strain was over 280,000-fold resistant to Cry1Ac protoxin (Zhao, unpublished data) but was susceptible to Cry1C-expressing broccoli (Cao et al. 1999). The Cry1C^R strain was originally collected in 1997. It was over 500-fold resistant to Cry1C protoxin but was susceptible to Cry1Ac (Zhao et al. 2001).

Assay procedures

Detached leaf bioassays were performed using neonate larvae of susceptible, Cry1A^R and Cry1C^R DBM. A leaf of each hybrid line as well as the parental plants was used for the bioassay of each type of DBM. Untransformed broccoli plants were used as controls. Ten neonate larvae of each type were placed on the leaf surface. Each leaf was placed in a Polar plastic cup maintained at 25 °C under a 16/8-h light/dark regime. All insect bioassays were performed in triplicate. Leaf damage (estimated visually) and larval mortality were scored after 3 days. For insect assays with whole plants, second instar larvae were used. Each of three leaves of a *cry1Ac+cry1C* plant were infested with five Cry1A^R larvae, and three other leaves of the same plant were each infested with 5 Cry1C^R larvae. Each leaf was enclosed within a plastic sandwich bag. Leaf damage and insect mortality were evaluated and larval weight-increase measured after 5 days.

Statistical analyses

Insect mortality and ELISA values were analyzed as a completely randomized design of analysis of variance (ANOVA) with two or three repeated measures. Means were compared by the least significant difference (LSD).

Results

Production of *cry1Ac+cry1C* hybrid broccoli plants

We attempted to pyramid *cry1Ac* and *cry1C* genes in same plant by making sexual crosses between *cry1Ac* and *cry1C* plants that had been well-characterized by molecular, genetic and insect assays (Metz et al. 1995a, b; Cao et al. 1999; Tang et al. 1999). Thus, the levels of gene expression and insect control in the parental lines were known. Crosses were made between maternal *cry1C* and paternal *cry1Ac* plants producing various levels of Cry1C or Cry1Ac protein. Many combinations produced little viable seed, so most of our work used the ones for which good quantities of hybrid seeds were available. A few seeds from the cross of Q23 (a high *cry1A* expresser) with H14 (a high *cry1C* expresser) were later obtained and examined in assays of protein levels and insect resistance.

Because most of the parental lines were heterozygous for the Bt gene, it was necessary to select for transgenic F1 progeny. Seeds from the crosses were germinated on medium containing hygromycin to select hygromycin-resistant progeny, and those also carrying the *cry1Ac* gene were then identified by PCR using *cry1Ac*-specific primers (data not shown). This approach gave more consistent and reliable results than the addition of kanamycin to the germination medium. Alternatively, PCR with gene-specific primers was used to confirm the presence of the *cry1Ac* and *cry1C* genes in plants that were resistant to both hygromycin and kanamycin in leaf-piece assays (data not shown). After this initial selection and characterization, we focused on four hybrid combinations for further RNA and protein analyses, and insect bioassays.

Expression of *cry1Ac* and *cry1C* genes in hybrid plants

To ascertain whether both Bt genes were expressed in plants containing both genes, Northern-blot analysis was performed (Fig. 2). Only the 1.9-kb *cry1C* mRNA band was detected in *cry1C* plants, and only the 3.6-kb c*ry1Ac* mRNA band was seen in *cry1Ac* plants although some non-specific bands associated with the *cry1Ac* hybridiza-

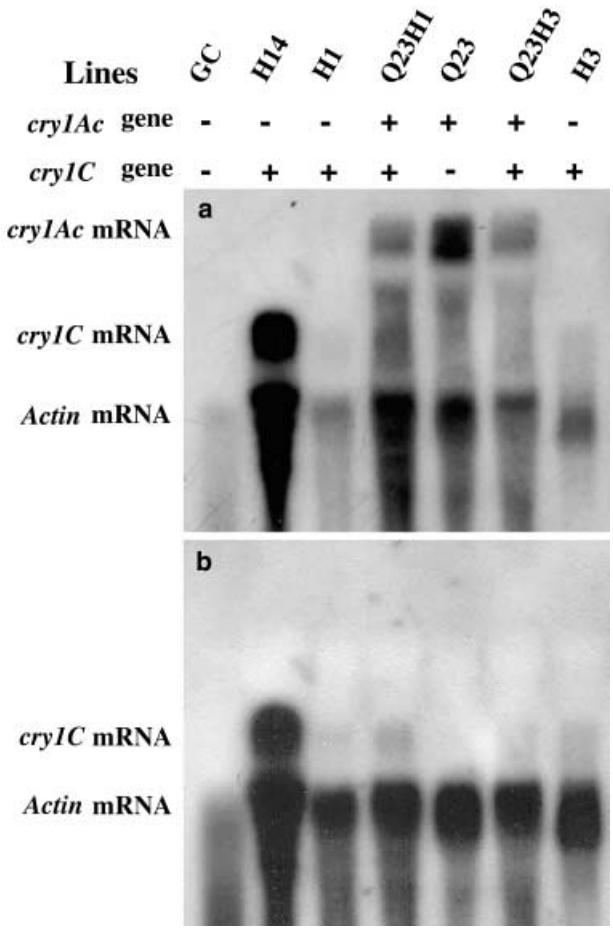

Fig. 2 Northern-blot analysis to detect the *cry1Ac* and *cry1C* mRNAs present in *cry1Ac+cry1C* hybrid plants. The RNA gel-blot was first hybridized with a ^{32}P-labeled 1.9-kb *Bam*HI fragment containing the entire *cry1C* gene, a labeled 1.3-kb PCR fragment containing part of the *cry1Ac* gene, and a 1.5-kb actin gene fragment from *Brassica*. The blot was then stripped and re-hybridized with the *cry1C* and actin probes

tion were seen in the Northern blot (Fig. 2a). Both bands were present in the *cry1Ac+cry1C* plants. No bands were visible in the untransformed control. These results indicate that steady state mRNA of both Bt genes stably accumulated in our hybrid plants and that the presence of both genes did not result in gene silencing.

Production of Cry1Ac and Cry1C proteins in hybrid plants

ELISA analysis showed that the hybrid plants analyzed all produced both Cry1Ac and Cry1C protein (Table 1). The amount of proteins varied, depending on the parental lines used. Line Q23 produced high levels of Cry1Ac protein, and hybrids in which Q23 was a parent produced a similar high level. On the other hand, line Q7, as well as its resulting hybrids, produced only a marginal level of the Cry1Ac protein. The same pattern was seen with the lines carrying the *cry1C* gene and producing Cry1C protein. Lines H14 and H12 produced high levels of Cry1C protein while H1 and H3 produced moderate or low levels, respectively. Hybrids from crosses using H14 or H12 as the female parent maintained a high level of Cry1C production while those using H1 or H3 produced moderate or low levels of the protein. Crosses of lines Q23 and H14, which produce high levels of Cry1Ac or Cry1C protein, respectively, resulted in hybrid plants with high levels of both proteins (Table 1).

To see whether the production of both Bt proteins was maintained in F_2 progeny plants, we selected ten hygromycin- and kanamycin-resistant F_2 progeny of the Q23 × H1 hybrid and performed ELISA analysis on them. Levels of production of the Cry1Ac and Cry1C proteins in the F_2 plants were consistent with the levels seen in the F_1 hybrid plants (data not shown).

Insect bioassays

To confirm that the Cry1Ac and Cry1C proteins produced in the hybrid plants were functional, isolated leaves were tested for their lethality to susceptible, Cry1A^R and Cry1C^R DBM (Table 2). After 3 days, larvae of all three

Table 1 Production of Cry1A and Cry1C proteins in Bt-transgenic broccoli plants evaluated by ELISA

Plant	Bt genes	Cry1Ac protein (ng/g)[a]	Cry1C protein (ng/g)[a]
Green Comet	–	0	0
Q23	*cry1Ac*	631 ± 23	0
Q7	*cry1Ac*	6 ± 3	0
H14	*cry1C*	0	1,122 + 117
H12	*cry1C*	0	1,092 ± 11
H1	*cry1C*	0	221 ± 50
H3	*cry1C*	0	66 ± 6
Q23 × H1	*cry1Ac+cry1C*	694 ± 23	267 ± 23
Q23 × H3	*cry1Ac+cry1C*	724 ± 48	76 ± 4
Q7 × H12	*cry1Ac+cry1C*	4 ± 1	1,226 ± 47
Q7 × H14	*cry1Ac+cry1C*	4 ± 1	1,100 ± 70
Q23 × H14-1[b]	*cry1Ac+cry1C*	801 ± 115	941 ± 168
Q23 × H14-2[b]	*cry1Ac+cry1C*	620 ± 119	1,101 ± 112
Q23 × H14-3[b]	*cry1Ac+cry1C*	680 ± 57	1,380 ± 51

[a] ng of Cry1Ac or Cry1C protein per g of fresh leaf tissue ± SE
[b] These hybrids were recently obtained from three independent crosses and assayed separately from the other plants

Table 2 Control of Cry1A^R or Cry1C^R neonate diamondback moth (DBM) larvae by detached leaves of *cry1Ac*, *cry1C* or *cry1Ac+cry1C* broccoli plants 3 days after infestation. Mortality values followed by different superscripts are significantly different (P<0.05)

Plant	Bt genes	Susceptible DBM		Cry1A^R DBM		Cry1C^R DBM	
		Defoliation (%)	Mortality (%)	Defoliation (%)	Mortality (%)	Defoliation (%)	Mortality (%)
Green Comet	–	40	0 ± 0^a	30	10 ± 0^a	30	13 ± 12^a
Q23	*cry1Ac*	0	100 ± 0^c	25	23 ± 15^a	3–5	87 ± 6^c
Q7	*cry1Ac*	25	33 ± 6^b	30	27 ± 12^a	20	40 ± 10^{ab}
H14	*cry1C*	0	100 ± 0^c	0	100 ± 0^b	30	37 ± 6^a
H12	*cry1C*	0	100 ± 0^c	0	100 ± 0^b	25	30 ± 10^a
H1	*cry1C*	0	100 ± 0^c	0	100 ± 0^b	15	30 ± 0^a
H3	*cry1C*	0	100 ± 0^c	0	100 ± 0^b	20	30 ± 10^a
Q23 × H1	*cry1Ac+cry1C*	0	100 ± 0^c	0	100 ± 0^b	0–5	90 ± 10^c
Q23 × H3	*cry1Ac+cry1C*	0	100 ± 0^c	0	100 ± 0^b	0–5	90 ± 10^c
Q7 × H12	*cry1Ac+cry1C*	0	100 ± 0^c	0	100 ± 0^b	10	70 ± 10^c
Q7 × H14	*cry1Ac+cry1C*	0	100 ± 0^c	0	100 ± 0^b	15	67 ± 12^{bc}
Q23 × H14-1*	*cry1Ac+cry1C*	0	100 ± 0^c	0	100 ± 0^b	0	100 ± 0^c

* This hybrid was assayed later than the others, using a more advanced Cry1C^R DBM population. The earlier Cry1C^R DBM population contained about 10% Cry1A^R insects while the later one did not. This may explain why the earlier assays with Q23 materials showed less than 100% mortality of the Cry1C^R population, even though these plants had high expression of Cry1A protein

DBM lines caused severe damage on leaves from an untransformed control plant. There was little or no insect mortality, and most larvae advanced to the second instar. Leaves of the four *cry1C* lines caused 100% mortality of susceptible and Cry1Ac^R neonates but were severely damaged by Cry1C^R larvae. This was true regardless of whether the line produced high, moderate, or low levels of Cry1C protein. Similarly, leaves from *cry1Ac* line Q23, which produced high levels of Cry1Ac protein, were highly toxic to susceptible or Cry1C^R larvae but not to Cry1A^R larvae. In contrast, when Cry1A^R or Cry1C^R neonates were placed on leaves from Q23 × H1 or Q23 × H3 hybrids producing both Cry1C and Cry1Ac proteins, they stopped feeding and most or all were dead within 48 h. Little or no leaf damage was seen. As expected, bioassay of a Q23 × H14 hybrid plant producing high levels of both Cry1Ac and Cry1C proteins resulted in 100% mortality of all three types of DBM larvae with no defoliation (Table 2). Line Q7 and its resulting hybrids, which produced only marginally detectable levels of Cry1Ac protein, suffered severe defoliation and did not completely control Cry1C^R DBM.

To confirm the effectiveness of the Cry1Ac and Cry1C proteins in whole plants, three leaves of a Q23 × H1 hybrid plant, together with its parents and an untransformed control, were infested with late second instar Cry1A^R larvae, and three other leaves were infested with Cry1C^R larvae. After 5 days, all control leaves were severely to completely defoliated by both types of larvae (Fig. 3). The average body weight per larva increased by 4 mg (400%) during this time period. The surviving larvae advanced to the fourth instar, and some had pupated. Leaves of *cry1Ac* or *cry1C* plants exhibited high toxicity to Cry1C^R or Cry1A^R larvae but were unable to control Cry1A^R or Cry1C^R ones, respectively (Fig. 3). In contrast, *cry1A+cry1C* hybrid plants showed little or no feeding damage from either DBM population (Fig. 3) and caused 100% mortality of both types of larvae.

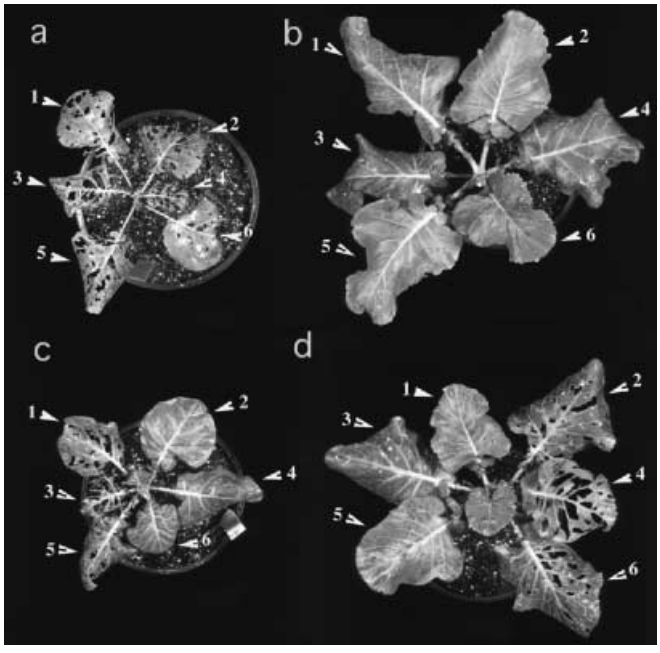

Fig. 3 Bioassay of intact *cry1Ac*, *cry1C* and *cry1A+cry1C*, and untransformed broccoli plants for control of late second instar Cry1A^R and Cry1C^R diamondback moth larvae. Each odd-numbered leaf was infested with five Cry1A^R larvae and each even-numbered leaf with five Cry1C^R larvae. Larvae were allowed to feed for 5 days. *a*: Green Comet control; *b*: *cry1Ac+cry1C* hybrid (Q23 × H1); *c*: *cry1Ac* plant (Q23); *d*: *cry1C* plant (H1)

Discussion

Only a few reports are available on the pyramiding of multiple foreign genes in the same variety for pest management. The strategies employed to-date include use of a binary construct carrying two or more insect resistance genes for plant transformation (Tian et al. 2000), sequen-

tial transformation of plants with two different insect resistance genes (Greenplate et al. 2000), or co-transformation with two binary vectors, each of which carries an insect resistance gene (Hua et al. 1993). In order to study the effects of two different Bt genes on insect control and the delay of insect resistance, we made sexual crosses between *cry1C* and *cry1Ac* broccoli plants. The parental plants were well-characterized in terms of the number of introduced genes and the levels of gene expression; therefore, the outcome in progeny plants could be compared to the parental material. Selection of parental plants for crosses allowed us to produce hybrid lines producing Bt proteins at desirable and co-ordinate levels for effectiveness in insect resistance management. Growth and development of the *cry1Ac+cry1C* hybrid plants were normal, as was seed production.

In previous studies with *cry1C* broccoli plants (Cao et al. 1999) we used Western-blot analysis to determine the level of Cry1C protein in the plants. Based on the intensity of bands on the Western blot we concluded that a high level of the Cry1C protein was present in H14 and H12 plants and a moderate or low level in H1 and H3 plants. In the current studies, ELISA was used to quantify the levels of Cry1C and/or Cry1Ac proteins. The two types of assay gave consistent results; however, ELISA provided more-accurate quantitative data and is more convenient for the measurement of a large number of samples.

Although multiple copies of genes and promoter sequences could result in the inhibition of gene expression in plants (Matzke et al. 1994; Meyer 1998) a number of studies have indicated that this does not always occur (Hobbs et al. 1990; Maqbool et al. 1999; Gahakwa et al. 2000). Kohli et al. (1999) showed that lines containing up to five copies of *gusA* and/or *bar* could express the gene stably at high levels through the R_3 generation. Greenplate et al. (2000) reported that inclusion of a second Bt gene had no deleterious effect on the level of production of the Cry1Ac protein. Our goal was to produce functional *cry1Ac+cry1C* hybrid broccoli plants: active and stable expression of both Bt genes and production of their proteins are essential for success of the pyramiding strategy. To increase the chances of achieving our goal, we made various cross combinations between diverse *cry1Ac* and *cry1C* parents. Molecular analyses of the hybrids tested showed that both genes were stably expressed in each cross combination at levels comparable to their parents. Crosses of parents with high levels of expression of one gene produced hybrid progeny with high expression of both genes. Furthermore, F_2 progeny from a Q23 × H1 hybrid showed that their levels of Cry1Ac and Cry1C proteins were consistent with those seen in the F_1 hybrid. Taken together, our results support the concept that researchers can successfully pyramid two closely related Bt genes into a single cultivar for resistance management.

Pyramiding two or more insecticidal genes in the same plant is a promising long-term strategy for delaying resistance, and one that is more forgiving on refuge size. Recent simulation modeling studies (Roush 1998) have predicted the number of generations required for insect populations to adapt to two resistance genes deployed either sequentially or pyramided in a single cultivar. These models showed that deployment of two insect resistance genes pyramided in a single cultivar was in general more durable than the sequential deployment of cultivars with single insect resistance traits. However, durability was also dependent on initial resistance gene frequency in the insect population, dominance of resistance, and epistasis (Gould 1998). If the insect resistance trait is recessive and the level of insect resistance is about the same in plants possessing one or both insect-resistant traits, then pyramided deployment is expected to be much more durable than sequential deployment. Availability of the *cry1Ac+cry1C* hybrid broccoli plants will allow us to test these models experimentally. By placing and monitoring susceptible, $Cry1A^R$ and $Cry1C^R$ DBM on *cry1Ac*, *cry1C* and *cry1Ac+cry1C* plants, we can select for $Cry1A^R$, $Cry1C^R$ or $Cry1C^R$+$Cry1A^R$ DBM in controlled settings, and learn modes and dynamics of the evolution of resistance to Cry1A and Cry1C during the selection.

The evolution of insect resistance is a major concern in agricultural use of Bt-crops, and appropriate resistance management strategies are needed to ensure its durability (Shelton et al. 2000; Tang et al. 2001). Currently, the "high-dose/refuge" strategy is favored with crops carrying a single Bt gene. A refuge is still essential for the durability of crops carrying two or more insect resistance genes even though the frequency of individual insects simultaneously resistant to two toxicants may initially be very low. However, with crops carrying pyramided Bt genes we expect that the required refuge size would be much reduced, although no experimental data is available to recommend the optimal refuge size for pyramided Bt crops. The *cry1Ac+cry1C* broccoli lines, in combination with our double-resistant DBM populations, are unique resources for further investigations on the effect of refuge size in greenhouse or field settings. Such studies could provide valuable information for the development of regulations related to the deployment of pyramided Bt crops.

In conclusion, we have produced broccoli plants containing both *cry1Ac* and *cry1C* genes. Both genes were actively expressed in these plants, and the resulting proteins were functional against both $Cry1A^R$ and $Cry1C^R$ DBM. These plants are valuable resources for further studies of insect resistance management.

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