

# Survival, Development, and Oviposition of Resistant Diamondback Moth (Lepidoptera: Plutellidae) on Transgenic Canola Producing a *Bacillus thuringiensis* Toxin

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**ABSTRACT** We measured responses of diamondback moth, *Plutella xylostella* L., to transgenic and nontransgenic canola, *Brassica napus* L. Transgenic canola expressed a *cry1Ac* gene of *Bacillus thuringiensis* Berliner resulting in  $238 \pm 29$  ng of Cry1Ac protein per milligram of total extractable protein in leaves. We tested 2 Hawaiian strains of diamondback moth: NO-QA was resistant to Cry1Ac and LAB-PS was susceptible. Larval and pupal durations, pupal weights, and adult emergence of the 2 strains were similar on nontransgenic canola, but differed significantly on transgenic canola. Transgenic canola killed all larvae tested from the susceptible strain. In contrast, for the resistant strain, no differences occurred between transgenic and nontransgenic canola in larval survival and head capsule width at day 5, percentage pupation, pupal weight, percentage adult emergence, and extent of defoliation. For both the susceptible and resistant strains of diamondback moth, no differences were detected between transgenic and nontransgenic canola in feeding initiation or oviposition preference. The lack of discrimination between transgenic and nontransgenic canola by neonates and ovipositing females indicates that host choice behavior is independent from susceptibility to Cry1Ac. Development of resistant diamondback moth on transgenic canola without any adverse effects provides an example of a pest that has completely overcome high levels of a *B. thuringiensis* toxin expressed by a genetically engineered plant.

**KEY WORDS** *Plutella xylostella*, *Bacillus thuringiensis*, *Brassica napus*, transgenic plants, resistance, transgenic canola

CROPS THAT HAVE been genetically engineered to express insecticidal proteins from the soil bacterium *Bacillus thuringiensis* Berliner include corn, cotton, and potato. In the United States during 1997, acreages occupied by *B. thuringiensis* toxin expressing varieties were 7 million for corn, 1.7 million for cotton, and 25,000 for potato (Mellon 1998). Generally these varieties have been successful in controlling the targeted pests. Naturally high levels of tolerance of cotton bollworm, *Helicoverpa zea* (Boddie), to Cry1Ac, which is the toxin expressed by transgenic cotton, contributed to false alarms that this pest had evolved resistance to transgenic cotton in 1996 (Stone and Sims 1993, Benedict et al. 1996, Kaiser 1996, MacIwain 1996, Greenplate 1997).

So far, no evidence has been reported that any pest has evolved resistance to *B. thuringiensis* toxin-expressing crops in the field. However, some pests have

evolved resistance to *B. thuringiensis* toxins in the laboratory (Tabashnik 1994a, Moar et al. 1995, Huang et al. 1997). Further, the diamondback moth, *Plutella xylostella* L., a global pest of cruciferous crops, has evolved resistance to foliar sprays of *B. thuringiensis* in numerous field populations (Tabashnik 1994a, Tabashnik et al. 1997b). Diamondback moth thus offers opportunities to understand and manage resistance to *B. thuringiensis* better.

The diamondback moth host plants—broccoli, *Brassica oleracea* L. (Metz et al. 1995) and canola, *Brassica napus* L. (Stewart et al. 1996b)—have been transformed with *cry1Ac* gene from *B. thuringiensis*. Although some potential exists for commercializing *B. thuringiensis* expressing-crucifers (Hokkanen and Wearing 1995), such plants are perhaps most useful in evaluating responses of resistant and susceptible strains of diamondback moth to gain insights about resistance. Because Cry1Ac is 1 of the toxins contained in a widely used spray formulation of *B. thuringiensis* subspecies *kurstaki* (Abbott Laboratories 1992), some resistance to Cry1Ac in diamondback moth strains resistant to *B. thuringiensis* subspecies *kurstaki* is expected. Indeed, resistance to Cry1Ac was documented in diamondback moth (Tabashnik et al. 1993), but until transgenic crucifers were tested, it was not

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known if the level of resistance was sufficient to enable the insect to overcome the *B. thuringiensis* toxins in transgenic plants.

Metz et al. (1995) found that a strain of diamondback moth that had evolved resistance to foliar sprays of *B. thuringiensis* subspecies *kurstaki* in the fields of Florida could survive and reproduce on transgenic broccoli that produced Cry1Ac. During 4 continuous generations of rearing on transgenic plants, Metz et al. (1995) observed that the resistant strain of diamondback moth defoliated the plants and showed rapid population growth. After the strain had been selected in the laboratory with a foliar *B. thuringiensis* product, subsequent tests showed that most, but not all, larvae from this strain could survive on leaves of transgenic broccoli (Tang et al. 1997). These results demonstrate that for 1 strain of diamondback moth, resistance to foliar *B. thuringiensis* treatments conferred resistance to transgenic plants.

To our knowledge, the papers by Metz et al. (1995) and Tang et al. (1997) are the only previously published reports of successful growth and reproduction of a *B. thuringiensis*-resistant strain of an insect on codon-optimized transgenic plants that kill 100% of a conspecific *B. thuringiensis*-susceptible strain. However, these studies did not report the concentrations of Cry1Ac in the transgenic plants used nor did they report the quantitative data that would enable rigorous comparison of survival, growth, feeding, and oviposition of resistant and susceptible strains on transgenic and nontransgenic plants.

Here we report detailed comparisons between responses to transgenic and nontransgenic canola by susceptible and resistant strains of diamondback moth from Hawaii.

### Materials and Methods

**Plants.** The canola cultivar 'Oscar' transformed with a synthetic, codon-optimized *cry1Ac* gene (transgenic line O52-6; Stewart et al. 1996b) (referred to as transgenic or Bt canola), previously reported to kill diamondback moth in all growth stages and under field conditions (Ramachandran et al. 1998b), was used in all the studies along with nontransformed Oscar (referred to as nontransgenic or NBt canola). Plants were grown as described by Ramachandran et al. (1998a). Plants were fertilized with a 0.25% solution of Peter's soluble fertilizer (20:20:20) 15 d after germination. We used plants 30–35 d old (6–8 leaf stage) for the experiments.

**Insects.** We studied 2 strains of diamondback moth from Hawaii—a resistant strain (NO-QA) and a susceptible strain (LAB-PS). The resistant strain was derived from a field population in Pearl City, Oahu, that had evolved moderate resistance to *B. thuringiensis* subspecies *kurstaki* in the field (Tabashnik et al. 1990) and was subsequently selected for extremely high levels of resistance to *B. thuringiensis* subspecies *kurstaki* (including Cry1Ac) in the laboratory (Tabashnik et al. 1997a). The LAB-PS strain is a susceptible isofemale line derived from LAB-P (Liu and B.E.T., unpublished

data). The LAB-P strain was started from a field population near Pulehu, Maui (Tabashnik et al. 1987). When the LAB-PS strain was started, LAB-P had been reared in the laboratory without exposure to any insecticide for >200 generations.

**Cry1Ac Concentration.** We measured Cry1Ac concentration in transgenic canola plants (30 d old) using western blots and immunostaining (Stewart et al. 1996a). Cry1Ac concentration was measured in 1 fully opened leaf (2nd from the top of the plant) from each of 24 individual transgenic plants grown in the greenhouse.

**Larval Survival and Development.** Single fully opened leaves (2nd from the top) of transgenic and nontransgenic plants were placed individually in petri plates (15 by 1.5 cm) lined with moistened filter paper. Each leaf was infested with 10 resistant or 10 susceptible diamondback moth neonates using a fine camel's-hair brush. The petri plates were sealed with parafilm to prevent moisture loss. New leaves were provided every 3 d until all surviving larvae pupated. On the 5th day, the number of larvae surviving on each plate was recorded. Weight and head capsule width of surviving larvae were measured on the 5th day. The pupae were weighed, placed individually in diet cups, and percentage of adult emergence was recorded. The experiment was replicated 6 times in a randomized complete block design in a laboratory under a photoperiod of 14:10 (L:D) h.

**Feeding Site Establishment.** A single freshly hatched resistant or susceptible diamondback moth larva was released on a fully opened transgenic or nontransgenic leaf placed in a petri plate (15 by 1.5 cm) lined with moistened filter paper. The larva was observed at 125 $\times$  for 1 min once in every 15 min for 60 min with a computer monitor using a software (Image-Pro) that was connected to a microscope (Olympus- binocular) through a solid state color video camera (Hitachi, model VK-C350). Larvae that ate leaf tissue and showed little or no net displacement during the 1-min observation period were scored as having established a feeding site. Larvae were grouped as establishing feeding sites in 0–15, 15–30, 30–45, 45–60 min, when a larva did not establish a feeding site in the first 60 min it was scored as having failed to establish a feeding site. Thirty-five larvae were observed for each diamondback moth strain on leaves of each plant type (total  $n = 140$ ).

**Feeding Damage.** In the free-feeding test, 2 transgenic and 2 nontransgenic plants were arranged closely with their foliage intermingling as a block. Each plant was infested with 25 resistant or 25 susceptible diamondback moth neonates. The blocks were separated from one another to prevent any larval movement between the blocks. In the restricted feeding test, each plant was infested with 15 resistant or 15 susceptible diamondback moth neonates and arranged singly without touching one another to restrict larval movement between plants. Twelve days after infestation, percentage defoliation of the plants was visually estimated. To avoid observer variation, only a single person estimated percentage of defoliation.

Table 1. Survival and pupal weight of the resistant and susceptible diamondback moth strains on transgenic and nontransgenic canola leaves

Strain	Plant type	% larval survival on day 5	% pupation	Pupal wt, mg	% adult emergence
NO-QA	Transgenic	93 ± 3aA	87 ± 5aA	7.5 ± 0.2aA	75 ± 6aA
NO-QA	Nontransgenic	95 ± 2aA	83 ± 5aA	7.6 ± 0.3aA	71 ± 6aA
LAB-PS	Transgenic	0bB	0bB	0bB	0bB
LAB-PS	Nontransgenic	90 ± 4aA	78 ± 5aA	7.5 ± 0.2aA	67 ± 3aA

Means followed by different lower case letters within a column indicate significant differences ( $P < 0.05$ ; LSD) between strains on leaves of different plant types. Different upper case letters indicate differences between plant types within a strain. NO-QA, resistant strain; LAB-PS, susceptible strain.

Both feeding tests were conducted in a greenhouse under natural light conditions in a randomized complete block design and replicated 5 times.

**Oviposition Preference.** Three transgenic and 3 nontransgenic plants were randomly arranged in a plastic tray (45 by 30 by 6 cm) filled with water to a depth of 2 cm. The plastic tray with the plants was then placed in a wooden cage (95 by 40 by 60 cm) covered with Saran mesh screen (25 by 25 cm). One-hundred resistant or 75 susceptible diamondback moth pupae (3 d old) were placed in a petri plate at the center of the cage. The adults were allowed to emerge, mate, and oviposit on plants within the cage. Eight days after pupae were introduced into the cages, plants were removed from the cage and the eggs laid on each plant were counted. This experiment was conducted in a laboratory under a photoperiod of 14:10 (L:D) h and replicated 5 times in a randomized complete block design.

**Data Analysis.** Responses of diamondback moth to transgenic and nontransgenic canola were analyzed using analysis of variance (ANOVA) (PROC GLM); means were separated using the protected least significant difference (LSD) test (SAS Institute 1985). A chi-square test was performed to identify differences between strains in feeding site initiation on transgenic and nontransgenic leaves. In free-feeding tests in which 2 plants of each entry were placed within a block, the means of 2 plants were analyzed. In the oviposition preference test, percentage of eggs laid on each plant within a block was calculated from the total number of eggs laid in that block. All the percentage data were transformed with an arcsine square-root transformation before analysis.

## Results

**CryIAc Concentration.** Transgenic plants had  $238 \pm 29$  (mean  $\pm$  SE) ng of CryIAc protein per mg of extractable protein. This was lower than the concentration reported previously for the T1 generation plants of the same transgenic line (Stewart et al. 1996b). We used T2 generation plants in our studies. Moreover, plants were grown in growth chambers in the earlier study (Stewart et al. 1996b), whereas in our experiments plants were grown in a greenhouse. This difference in growing conditions could have resulted in variation in the toxic protein concentration levels.

**Larval Survival and Development.** Transgenic canola killed all the susceptible strain larvae ( $n = 60$ ). In contrast, no significant differences were recorded for the resistant strain on transgenic and nontransgenic canola leaves for larval survival on day 5 ( $F = 0.04$ ;  $df = 1, 5$ ;  $P > 0.85$ ), percentage pupation ( $F = 0.19$ ,  $P > 0.68$ ), pupal weight ( $F = 0.08$ ,  $P > 0.78$ ), and percentage adult emergence ( $F = 0.12$ ,  $P > 0.75$ ) (Table 1). When observations of both diamondback moth strains were analyzed together, larval survival and adult emergence rates were not significantly different for the resistant strain on transgenic and nontransgenic leaves and for the susceptible strain on the nontransgenic leaves.

The resistant strain larvae grew and developed normally on both transgenic and nontransgenic leaves, whereas the susceptible larvae grew and developed normally only on nontransgenic leaves (Table 2). No significant differences were identified in head capsule width ( $F = 0.22$ ;  $df = 2, 10$ ;  $P > 0.81$ ) and body weight ( $F = 1.22$ ,  $P > 0.34$ ) for the surviving larvae of resistant strain exposed to transgenic and nontransgenic leaves and susceptible strain on nontransgenic leaves. The surviving larvae of both strains were late 2nd or early 3rd instars on both leaf types when measurements were made.

**Feeding Site Establishment.** For both the resistant and susceptible strains, no significant differences in the time required for establishment of feeding sites occurred between transgenic and nontransgenic leaves ( $F = 1.689$ ,  $df = 4$ ,  $P > 0.79$  for the resistant strain and  $F = 1.387$ ,  $P > 0.85$  for the susceptible strain) (Table 3). More than 70% of the resistant strain and 60% of the susceptible strain neonates established

Table 2. Growth of the resistant and susceptible diamondback moth strain larvae on transgenic and nontransgenic canola leaves after 5 d

Strain	Plant type	Head capsule width, mm	Larval wt, mg
NO-QA	Transgenic	0.59 ± 0.1a	5.2 ± 0.3a
NO-QA	Nontransgenic	0.58 ± 0.1a	5.7 ± 0.3a
LAB-PS	Transgenic	—	—
LAB-PS	Nontransgenic	0.58 ± 0.1a	5.1 ± 0.2a

Means followed by the same letter indicate no significant differences ( $P < 0.05$ ; LSD); —, data were not recorded because no larvae survived on this plant type. NO-QA, resistant strain; LAB-PS, susceptible strain.

Table 3. Percentage of resistant and susceptible diamondback moth neonates establishing feeding sites on transgenic and nontransgenic canola leaves at different periods of time

Time, min	NO-QA (n)		LAB-PS (n)	
	Transgenic leaf	Nontransgenic leaf	Transgenic leaf	Nontransgenic leaf
0-15	40.0 (14)	34.3 (12)	25.7 (9)	22.8 (8)
15-30	31.4 (11)	37.1 (13)	34.3 (12)	48.6 (17)
30-45	17.1 (6)	11.4 (4)	17.1 (6)	11.4 (4)
45-60	5.7 (2)	5.71 (2)	11.4 (4)	8.6 (3)
Failed to establish feeding site in 60 min	5.7 (2)	11.4 (4)	11.4 (4)	8.6 (3)

NO-QA, resistant neonates; LAB-PS, susceptible neonates; n, number of larvae establishing feeding sites.

feeding sites within the first 30 min on both transgenic and nontransgenic leaves. Most of the neonates established feeding sites on the upper surface of the leaf. Less than 12% of resistant and susceptible strain neonates failed to establish feeding sites within the first 60 min on both leaf types.

**Feeding Damage.** Within the resistant strain no significant differences were observed in percentage defoliation of test plants in both feeding tests ( $F = 2.94$ ;  $df = 1, 4$ ;  $P > 0.16$  for free-feeding test;  $F = 0.67$ ,  $P > 0.46$  for restricted feeding test). But the susceptible strain inflicted significantly higher defoliation to nontransgenic plants compared with the transgenic plants in both feeding tests ( $F = 164.69$ ;  $df = 1, 4$ ;  $P < 0.0002$  for free feeding test;  $F = 681.7$ ,  $P < 0.0001$  for restricted feeding test) (Table 4). However, defoliation was not significantly different among transgenic and nontransgenic plants defoliated by the resistant strain and nontransgenic plants defoliated by the susceptible strain. Indeed, no measurable defoliation for the susceptible strain occurred on transgenic plants in the restricted feeding test, but a small amount of defoliation of transgenic plants occurred in the free feeding test presumably because larvae that developed on nontransgenic plants moved to transgenic plants to cause that little damage.

**Oviposition Preference.** No significant differences occurred in the percentage of eggs laid on transgenic and nontransgenic plant types for both resistant ( $F = 0.16$ ;  $df = 1, 4$ ;  $P > 0.71$ ) and susceptible strains ( $F = 0.08$ ,  $P > 0.79$ ) (Fig. 1).

Table 4. Percentage defoliation of transgenic and nontransgenic canola plants by resistant (NO-QA) and susceptible (LAB-PS) diamondback moth larvae in feeding damage tests

Strain	Plant type	% defoliation/plant	
		Free-feeding test	Restricted feeding test
NO-QA	Transgenic	17.0 ± 2.1aA	16.6 ± 1.9aA
NO-QA	Nontransgenic	21.6 ± 3.1aA	14.4 ± 1.1aA
LAB-PS	Transgenic	0.2 ± 0.2bB	0bB
LAB-PS	Nontransgenic	23.4 ± 3.1aA	15.8 ± 1.1aA

Means followed by different lower case letters within a column indicate significant differences ( $P < 0.05$ ; LSD) between strains on leaves of different plant types. Different upper case letters indicate differences between plant types within a strain.

## Discussion

The lack of significant differences for the resistant strain on transgenic and nontransgenic plant types for all the parameters tested clearly demonstrates the ability of the resistant strain to survive and develop successfully on transgenic canola plants synthesizing levels of the toxic protein that were lethal to a susceptible strain. Furthermore, the resistant strain inflicted damage to both transgenic and nontransgenic plants equally. This shows that the *cryIAC* gene in transgenic canola did not offer any protection against the resistant diamondback moth strain. But the same transgenic canola killed all larvae tested from the susceptible strain. Moreover, very low levels of damage to transgenic canola in the free-feeding test by the sus-

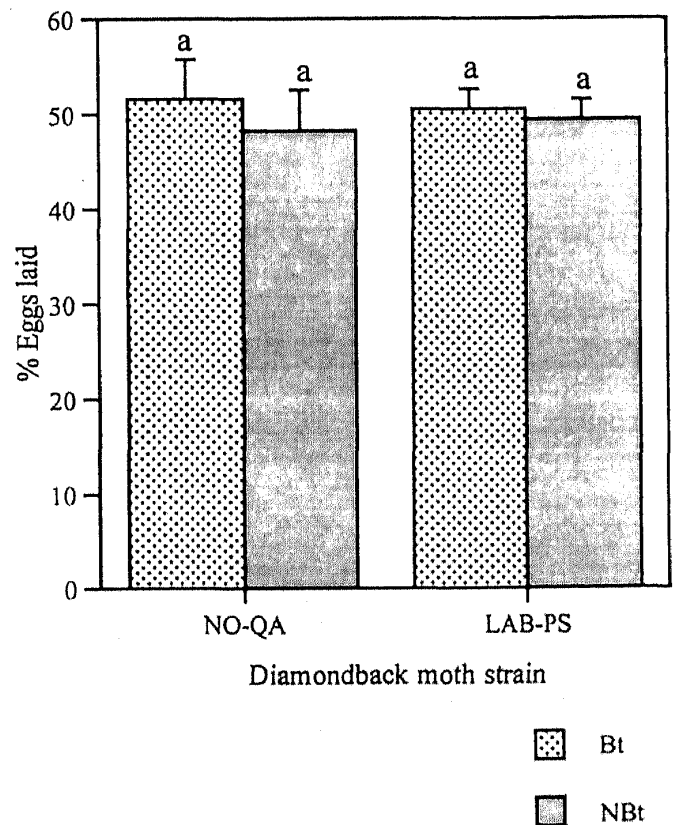


Fig. 1. Percentage of eggs laid by resistant (NO-QA) and susceptible (LAB-PS) diamondback moth strains on transgenic (Bt) and nontransgenic (NBt) canola plants in an oviposition preference test. Bars followed by the same letter within a strain are not significantly different ( $P > 0.05$ ; LSD).

ceptible strain indicates that even if susceptible larvae develop on the nontransgenic plants in their early instars and later move to the transgenic plants, susceptible larvae would not cause much damage to the transgenic plants. In an earlier study, 2nd, 3rd, and 4th instars of diamondback moth were observed to move from the infested plant within 24 h (Ramachandran et al. 1998a).

In a related study, a diamondback moth strain derived from the fields in Florida showed 25% survival on canola expressing a *cry1Ac* gene or *cry1Ac* plus potato proteinase inhibitor II genes where only the late larval instars were exposed to transgenic canola for a short duration (Winterer and Bergleson 1996). Furthermore, the toxic protein concentration in those transgenic plants was low (20–100 ng/mg of total protein) compared with the transgenic plants used in our study. Another diamondback moth population established from Florida was reported to complete its life cycle and damage a transgenic broccoli- expressing *cry1Ac* gene (Metz et al. 1995). Although the toxic protein expression levels in those transgenic broccoli plants have not been reported, they provided 100% control of a susceptible diamondback moth strain. The colony we tested was collected and developed from a Hawaiian population, and resistance to Cry1Aa, Cry1Ab, Cry1Ac, and Cry1F toxins was conferred by a single gene mutation in this colony (Tabashnik et al. 1997a). *B. thuringiensis* resistance in this colony also has been shown to be similar to the resistance in populations from Florida (Tabashnik et al. 1997b).

It was not surprising that no significant differences were identified for both strains in the percentage of larvae establishing feeding sites on transgenic and nontransgenic leaves. This shows that the neonates of both strains did not discriminate between nontransgenic and transgenic leaves containing the *cry1Ac* gene. Furthermore, lack of differences between strains in the feeding behavior of neonates suggests that there is no behavioral resistance in neonates against Cry1Ac toxin present in transgenic leaves, at least up to the point of establishment of feeding sites. An earlier study found no evidence of behavioral resistance in diamondback moth against spray formulations of *B. thuringiensis* (Schwartz et al. 1991).

The lack of significant differences in the percentage of eggs laid between transgenic and nontransgenic plant types shows that diamondback moth adults did not exhibit any discrimination for laying eggs on the 2 plant types. Furthermore, this also suggests that Cry1Ac toxin from the transgenic plants failed to deter oviposition by adults of the susceptible strain, which indicates that susceptibility of the larvae and oviposition preference by the adults are unrelated. In a similar study, diamondback moth adults failed to discriminate between cabbage leaf disks treated with *B. thuringiensis* spray formulations from untreated disks (Groeters et al. 1992). In another study, no significant differences were observed in the number of eggs laid by the European corn borer, *Ostrinia nubilalis* (Hübner), on nontransgenic and a transgenic corn containing *cry1Ab* gene (Orr and Landis 1997). These results

suggest that *B. thuringiensis* toxins in transgenic plants do not affect the oviposition behavior of adults.

Our results confirm and extend those of Metz et al. (1995). Transgenic canola leaves with high concentrations of Cry1Ac killed all susceptible larvae, but had no adverse effects on survival, development, and consumption by larvae from the resistant strain. For both susceptible and resistant strains examined, feeding initiation by neonates and adult oviposition preference did not differ between transgenic and nontransgenic canola.

In conjunction with previously published studies, the results reported here have some important implications for resistance management. First, discrimination between transgenic and nontransgenic varieties of a crop by ovipositing females might be achieved by altering the timing of planting or other factors (Alstad and Andow 1995); available data suggest that oviposition preference and feeding initiation by neonates are not affected by *B. thuringiensis* toxins. Therefore, unless specific data show otherwise, it seems reasonable to assume that oviposition and feeding initiation by neonates are distributed randomly between transgenic and nontransgenic varieties. Second, the results reported here and previously (Metz et al. 1995, Tang et al. 1997) show that resistant diamondback moth can completely overcome Cry1Ac toxin in transgenic crucifers that kill susceptible larvae. This type of resistance may reduce the tendency for nonrandom mating of resistant and susceptible adults, which might occur if partial resistance to transgenic plants caused differences in the development rate or size of resistant insects on transgenic plants compared with susceptible insects on nontransgenic plants. Synchronous emergence of resistant and susceptible adults would tend to reduce the likelihood of assortative mating, and thus enhance the ability of nontransgenic plant refuges to slow evolution of resistance (Tabashnik 1994b). Conversely, the absence of any adverse effects of transgenic plants on resistant insects would tend to increase the advantage of resistant genotypes, which could accelerate the evolution of resistance.

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