Transgenic *Bt*-producing *Brassica napus*: *Plutella xylostella* selection pressure and fitness of weedy relatives

Peter MASON[1],*, Lorraine BRAUN[2], Suzanne I. WARWICK[1], Bin ZHU[3], and C. Neal STEWART Jr.[4]

[1] Agriculture and Agri-Food Canada, Eastern Cereal and Oilseed Research Centre, K. W. Neatby Bldg., Ottawa, Ontario, K1A 0C6, Canada

[2] Agriculture and Agri-Food Canada, Saskatoon Research Centre, 107 Science Place, Saskatoon, Saskatchewan, S7N 0X2, Canada

[3] Environment Canada, National Water Research Institute, 11 Innovation Blvd., Saskatoon, Saskatchewan, S7N 3H5, Canada

[4] Dept. of Plant Sciences and Landscape Systems, 2431 Centre Drive, Ellington Plant Sciences, University of Tennessee, Knoxville, TN 37996-4561, USA

Release of transgenic insect-resistant crops creates the potential not only for the insect pest to evolve resistance but for the escape of transgenes that may confer novel or enhanced fitness-related traits through hybridization with their wild relatives. The differential response of diamondback moth (*Plutella xylostella*) populations in eastern and western Canada to *Bt*-producing (GT) *Brassica napus* and the potential for enhanced fitness of GT *B. napus* and weedy GT *Brassica rapa* × *B. napus* hybrid populations (F_1, BC_1, BC_2) were studied. Comparative bioassays using neonates and 4th instars showed that GT *B. napus* and GT *B. rapa* × *B. napus* hybrids are lethal to larvae from both populations. No measurable plant fitness advantage (reproductive dry weight) was observed for GT *B. napus* (crop) and GT *B. rapa* × *B. napus* hybrid populations at low insect pressure (1 larva per leaf). At high insect densities (>10 larvae per leaf), vegetative plant weight was not significantly different for GT *B. napus* and non-GT *B. napus*, whereas reproductive plant weight and proportion of reproductive material were significantly higher in GT *B. napus*. Establishment of the *Bt* trait in wild *B. rapa* populations may also increase its competitive advantage under high insect pressure.

Keywords: transgenic crops / *Brassica napus* / oilseed rape / canola / *Brassica rapa* / environmental effects / *Plutella xylostella* / diamondback moth / *Bt* / *Bacillus thuringiensis*

INTRODUCTION

Worldwide the use of transgenic crops has become an important component of pest management programs. In 2001, herbicide-resistant crops made up 77% of global commercial transgenic production, insect resistance (*Bacillus thuringiensis* Berliner, *Bt*) constituted 15% and stacked genes for herbicide and insect resistance (cotton and maize) made up 8% (James, 2001). In Canada, *Bt*-producing varieties constituted more than 35% of the corn planted in 2001 (Canadian Corn Pest Coalition, 2001).

Although no commercial lines of *Bt*-producing *Brassica napus* L. have been released, experimental transgenic *Bt*-producing *B. napus* lines have been generated that confer selective advantage in the presence of *Brassica*-defoliating insects, including diamond back

moth (*Plutella xylostella* L.), cabbage looper (*Trichoplusia ni* Hübner) and corn earworm [*Helicoverpa zea* (Boddie)] (Halfhill et al., 2001; Stewart et al., 1996, 1997; Ramachandran et al., 1998a). A number of transgenic *Bt*-producing *Brassica* vegetable crops are also being developed, including broccoli (*B. oleracea* L. var. *botrytis*) (Cao et al., 1999; Metz et al., 1995), cabbage (*B. oleracea* var. *capitata*) (Jin et al., 2000), and Chinese cabbage (*B. rapa* L. subsp. *chinensis*) (Cho et al., 2001; Xiang et al., 1999), using the *Bt cry1Ac* gene designed to control diamondback moth.

Diamondback moth is a pest of global significance in a variety of cruciferous crops. In Canada, it is a pest of vegetable crops (Howard et al., 1994) and populations routinely infest canola (cultivated varieties of either

* Corresponding author:
Tel.: (613) 759-1908; fax: (613) 759-1701; e-mail: masonp@agr.gc.ca

B. napus or *B. rapa* containing low erucic acid and glucosinolate levels, *B. napus* is also known as oilseed rape) in both the east and the west. In most years diamondback moth causes minor economic damage, but in some years populations reach very high densities and enormous crop losses occur. For example, in western Canada in 1995 more than 1.25 million ha of canola were sprayed with insecticide to control diamondback moth at an estimated cost to producers of $45 to 52 million (Western Committee on Crop Pests, 1995). Even larger populations occurred in 2001, and 1.8 million ha were treated with insecticide at a cost of ca. $72 million (Western Committee on Crop Pests, 2001). Diamondback moths do not normally overwinter in Canada (Butts, 1979; Dosdall et al., 2003; Harcourt, 1960) and pest populations originate primarily from the southern USA and Mexico (Dosdall et al., 2003; Philip and Mengersen, 1989; Putnam and Burgess, 1977). If migration occurs early in spring, populations can build to very high numbers over the summer, as the generation time is less than a month. If larval densities are high at susceptible plant stages (*i.e.* cabbage plants are small or canola pods green and with little green leaf material left) yield can be severely affected. Development of *Bt*-producing varieties could provide an important pest management tool for diamondback moth control in *Brassica* crops in Canada.

An important environmental concern of the release of transgenic insect resistant crops is the potential for the insect pest to evolve resistance. Several pest Lepidoptera have evolved resistance to *Bt* toxins in laboratory selection experiments (Tabashnik, 1994). Diamondback moth is the only insect to have evolved resistance to *Bt* when applied as a biopesticide in the field (reviewed in Ferré and van Rie, 2002), with resistant strains reported from several countries (Perez and Shelton, 1997; Shelton et al., 1993; Tabashnik, 1994; Tabashnik et al., 1997a, 1997b). Metz et al. (1995) observed a strain of diamondback moth that had evolved resistance to foliar sprays of *Bt* in fields and could survive on transgenic broccoli that produced the *Bt* cry1Ac protein. The spread of transgene-mediated pesticides and associated evolution of resistance could reduce the effective lifetime of these compounds (McGaughey et al., 1998).

Another major concern about the agricultural release of transgenic crops is the escape of transgenes that may confer novel or enhanced fitness-related traits into the environment through hybridization with their wild relatives (Metz et al., 1995; Snow, 2002; Warwick et al., 1999). *Brassica napus* is of particular concern, as it is a partially outcrossing species, forms volunteer (feral) populations and has numerous wild relatives present in cultivated areas in Canada and worldwide (Warwick et al., 2003). One such relative is bird's rape (*Brassica rapa* L.), which has a limited distribution as an agricultural and/or ruderal weed in *B. napus*-growing areas in eastern Canada (Quebec). In western Canada, *B. rapa* grown as canola also produces volunteer populations (Warwick et al., 2000). Spontaneous hybridization (Jørgensen and Andersen, 1994; Landbo et al., 1996) and introgression (Hansen et al., 2001) were reported between *B. napus* and wild *B. rapa* in Danish field studies, between both wild and cultivated lines of *B. rapa* and *B. napus* in field experiments in Canada (Bing et al., 1996; Warwick et al., 2003), and between wild *B. rapa* and *B. napus* in commercial canola fields in eastern Canada (Warwick et al., 2003). There is, therefore, a potential for *B. rapa* to acquire transgenes from *B. napus* through interspecific hybridization. Because important *Brassica* herbivore pests, such as diamondback moth, are also found on weedy crucifer populations (Warwick et al., 2000, 2002), there is the potential for development of enhanced fitness against insect attack in wild *B. rapa* populations.

The possibility for increased fitness of transgenic wild relative × crop hybrid and backcross generations depends on the nature of the transgene (Halfhill et al., 2002). Herbicide resistance is likely to confer less fitness advantage to wild populations than disease or insect resistance (Kareiva et al., 1996; Stewart et al., 1997). The insecticidal *Bt* transgene in a weed host could alter its natural ecology by giving the weeds a selective advantage as the result of natural insect pressure (Stewart et al., 1997). One of the first demonstrations of enhanced fitness was in a field study of *Bt*-producing *B. napus* under moderate insect selection pressure by diamondback moth (Stewart et al., 1997). Similarly, a *Bt* transgene that reduces insect herbivory in cultivated sunflowers (*Helianthus annuus* L.) also does so in wild sunflowers, leading to increased fecundity in one of the two sites studied (Snow et al., 2003).

To evaluate differential response of populations of diamondback moth in eastern and western Canada and to evaluate the risk of transgene flow into wild relatives, the following experiments were performed with *B. napus* engineered to express green fluorescent protein and *Bt* (GT) and weedy GT *B. rapa* × *B. napus* hybrid populations: (1) comparative bioassays using neonates hatched from eggs and 4th instars of eastern and western diamondback moth populations; and (2) evaluation of the effect of selection pressure from diamondback moth on plant fitness (reproductive success) of GT *B. napus* (crop) and GT *B. rapa* × *B. napus* hybrid populations (first
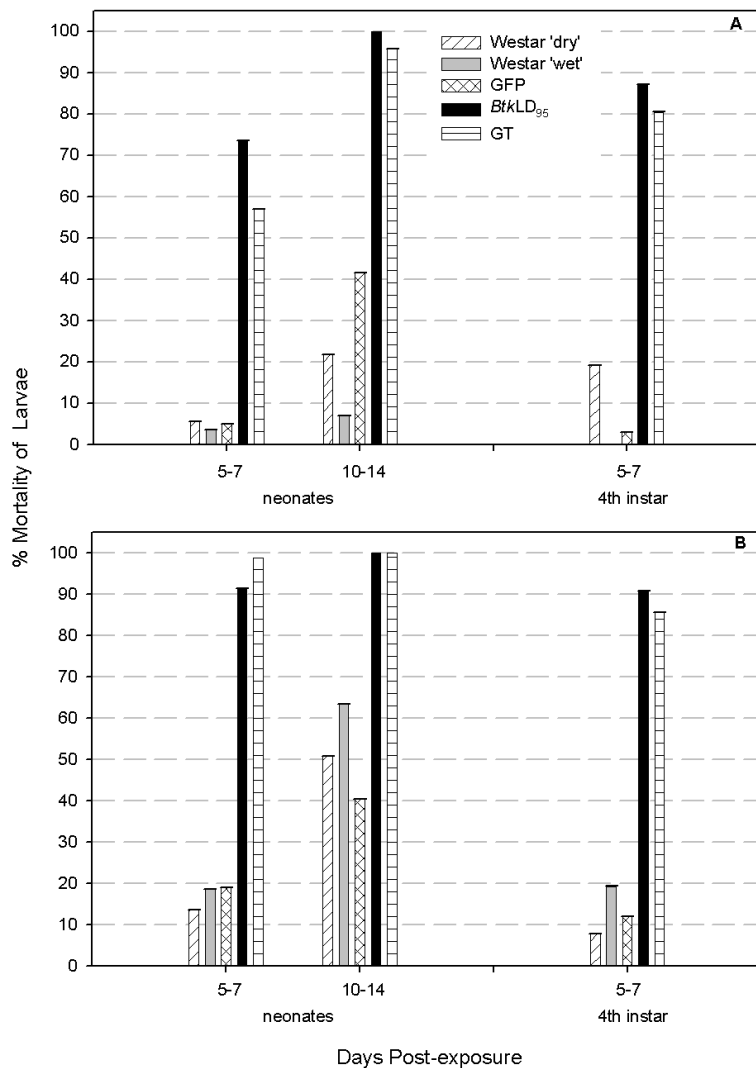
Figure 1. Mortality (%) of neonate (hatched from eggs) and 4th instar diamondback moth fed leaf disks from *Brassica napus* transformed with GFP or GFP and *Bt* (GT), or non-transformed *B. napus* cv Westar leaf disks which were untreated ('dry'), or dipped in water ('wet') or dipped in *Btk* LD_{95}: **A** eastern population; **B** western population.

generation progeny [F_1], parent × F_1 cross [BC1], parent × BC1 [BC_2]) under Canadian field conditions.

RESULTS

Laboratory insect bioassays

For neonate bioassays, egg hatch was generally >95% and occurred within 1–2 days of the start of experiments. Replicates where eggs did not hatch were not included in the analyses. For bioassays using 4th instars, only those larvae that consumed leaf material were included.

Brassica napus

Mortality of both eastern and western neonate and 4th instar diamondback moth fed leaf disks of GT *B. napus*

or non-GT *B. napus* treated with *Btk* was significantly higher than those fed untreated non-GT *B. napus* (Westar, dry, wet) or green fluorescent protein (GFP) expressing *B. napus* (Fig. 1). The high mortality of western neonates in the non-transgenic *B. napus* group occurred only after the first week of the experiment, prior to that mortality was approximately 20% compared with more than 90% in the GT *B. napus* or *Btk* LD_{95} treatment groups (Fig. 1B). In contrast to the 100% mortality observed for neonates, not all 4th instars died, survival being almost 27% in one case.

Brassica rapa

Designations of the various *B. rapa* populations and crosses used in experiments are shown in Table 1.

Table 1. Population source, designation, and line or cross of F_1 and backcross lines of *Brassica rapa* used in experiments.

Source	Designation	Line/Cross
Irvine, California	CA	wild population
	CA-BC$_1$	CA × (CA × GT2)
	CA-BC$_2$ a	CA × (CA × (CA × GT2))
	CA-BC$_2$ b	CA × (CA × (CA × GT5))
Milby, Quebec	QB1	wild population
	QB1-F$_1$	(QB1 × GT-2)
	QB1-BC$_1$	QB1 × (QB1 × GT-2)
	QB1-BC$_2$ a	QB1 × (QB1 × (QB1 × GT2))
	QB1-BC$_2$ b	QB1 × (QB1 × (QB1 × GT5))
Waterville, Quebec	QB2	wild population
	QB2-F$_1$	(QB2 × GT9)
	QB2-BC$_1$	QB2 × (QB2 × GT6)
	QB2-BC$_2$ a	QB2 × (QB2 × (QB2 × GT2))
	QB2-BC$_2$ b	QB2 × (QB2 × (QB2 × GT5))

Mortality of eastern neonate diamondback moth fed leaf disks of GT *B. rapa* × *B. napus* hybrid and backcross plants or non-GT *B. rapa* treated with *Btk* was higher than those fed untreated non-GT *B. rapa* plants for all three *B. rapa* populations studied (Fig. 2). At 5-7d mortality of neonates was 100% for all GT *B. rapa* plant types compared with <15% for control groups. For 4th instars mortality varied among GT *B. rapa* plant types (Fig. 2B–D). However, significantly more 4th instars fed GT *B. rapa* or non-GT *B. rapa* treated with *Btk* LD$_{95}$ died than those fed non-GT *B. rapa* in all but one case. The only exception was for QB1-BC$_2$b which was not significantly different from QB1 wet and dry controls (Fig. 2C). Although western neonates and 4th instars were evaluated on only the CA *B. rapa* population, the results clearly show that the effects of consuming GT *B. rapa* are the same as for eastern diamondback moth (Fig. 2E). The lethal effects of the *Bt* trait in *B. rapa* × *B. napus* F$_1$ hybrids and first and second backcross generations were the same. Furthermore there were no (or slight in the case of QB2 mortality at 18-21d) differences in mortality or amount of leaf material consumed between *B. rapa* populations or between *B. rapa* and *B. napus* (data not shown).

Mortality among GT *B. rapa* treatments ranged from less than 20% (eastern population, CA-BC$_2$b) to 100% (only four of the 11 treatments). Plant consumption by 4th instars offered GT *B. rapa* was similar for the three *B. rapa* populations (CA, QB1, QB2), significantly less than for larvae offered non-GT *B. rapa*, and was similar to consumption for GT *B. napus* (data not shown).

Field plant fitness trials

In 2001, field cage trials in both eastern and western locations supported low diamondback moth densities (approximately 1 larva per leaf) and showed that although significantly more leaf damage occurred to non-GT *B. napus* (18–24%) and non-GT *B. rapa* (22–30%) than to GT *B. napus* (0%) or GT *B. rapa* (<1%), larval feeding had no significant effect on the amount of vegetative or reproductive plant material produced (Tabs. 2 and 3). At the eastern location just before harvest, aphids infested a number of the cages and destroyed the plants inside, resulting in a reduction of the number of replicates for some of the treatments (Tab. 3).

In 2002, where Saskatoon field cage trials yielded higher diamondback moth densities (>10 larvae per leaf), GT *B. napus* showed significantly less leaf damage (0%) than non-GT *B. napus* (74%) and GFP *B. napus* (78%). While vegetative plant weight was not significantly different, reproductive plant weight and proportion of reproductive material were significantly higher for GT *B. napus* than for non-GT *B. napus* (Tab. 4).
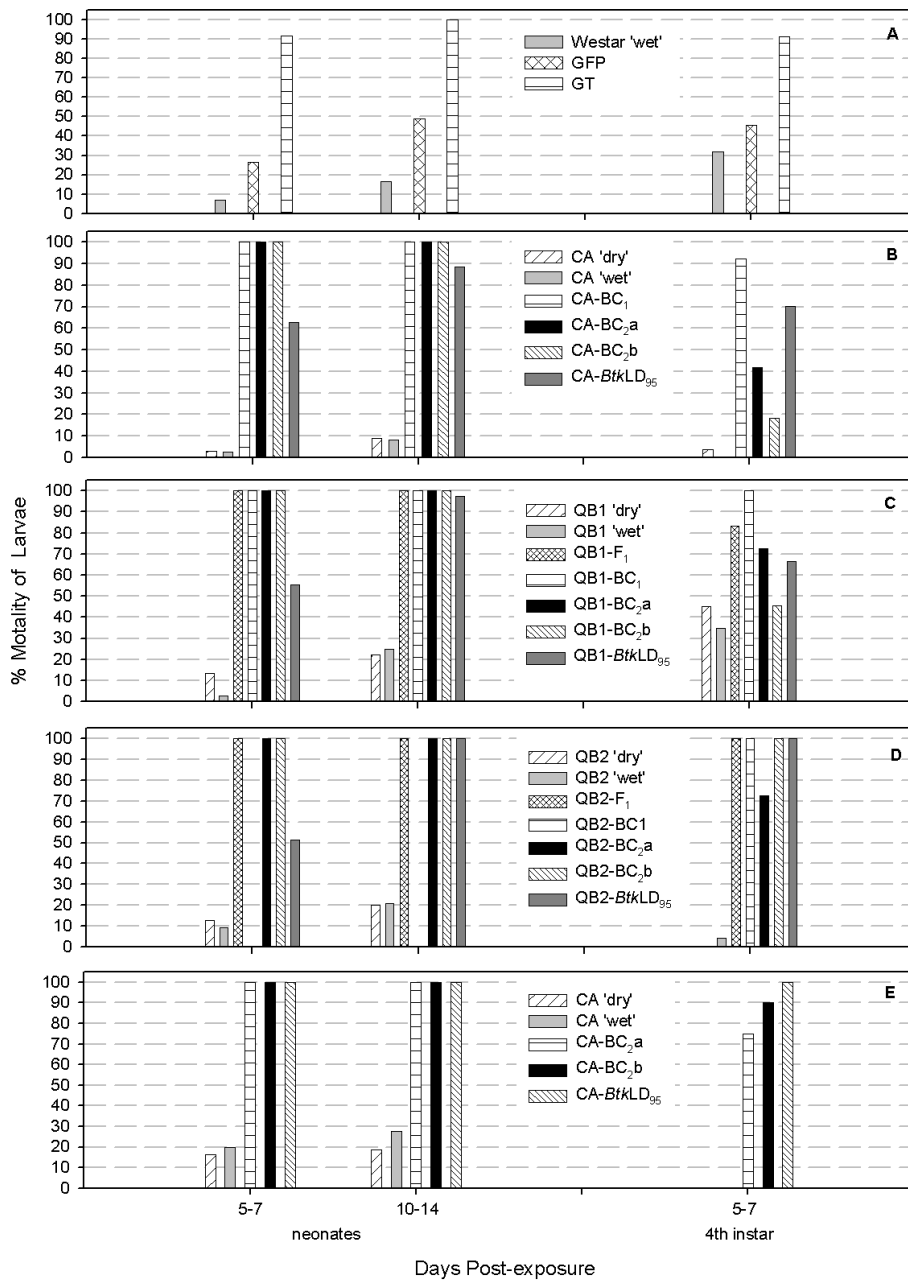
Figure 2. Mortality (%) of eastern (**A-D**) and western (**E**) neonate and 4th instar diamondback moth fed leaf disks from *Brassica napus* transformed with GFP and *Bt* (GT *B. napus* lines), and non-transformed leaf disks from *B. napus* cv Westar which were dipped in water ('wet') or from GFP-*Bt* transformed *B. rapa* first back-cross with parental line (BC_1) and second back-cross with parental line (BC_2), and non-transformed *B. rapa* California (CA) and Quebec (QB1, QB2) population leaf disks which were untreated ('dry'), dipped in water ('wet') or in the LD_{95} rate of Safer's BTK: **A** *B. napus* cv Westar; **B** California (CA) *B. rapa*; **C** Quebec (QB1) *B. rapa*; **D** Quebec (QB2) *B. rapa*; and **E** California (CA) *B. rapa*.

Table 2. Mean plant weight (± SE) (vegetative and reproductive components, and reproductive component expressed as proportion of total plant weight) of *Brassica napus* plants transformed with GFP and *Bt* (GT *B. napus* lines), and non-GT *B. napus* cv Westar grown under zero or low insect pressure from diamondback moth, *Plutella xylostella*, in Saskatoon in 2001. Values shown are mean individual weight per plant for 10 plants harvested separately per replicate, and mean total cage plant weight based on 40 plants per cage.

| | Mean Individual Plant Weight (± SE) | | | | | |
| Line | Vegetative | | Reproductive | | Reproductive as % of total | |
	zero†	low	zero	low	zero	low
Westar	6.5 ± 0.8 b	5.6 ± 0.6 a	11.0 ± 1.6 a	9.2 ± 1.2 b	56.9 ± 2.7 a	56.7 ± 2.7 bc
GFP	7.1 ± 1.0 b	5.1 ± 0.7 a	13.2 ± 2.0 a	10.1 ± 1.6 b	64.0 ± 1.5 a	61.4 ± 2.2 ab
GT 1	8.6 ± 0.9 ab	8.0 ± 1.1 a	17.5 ± 2.0 a	15.2 ± 2.4 ab	63.5 ± 1.9 a	61.3 ± 1.8 ab
GT 4	6.7 ± 0.8 b	6.9 ± 0.8 a	15.5 ± 2.1 a	13.9 ± 2.1 ab	62.9 ± 2.8 a	56.4 ± 3.3 c
GT 5	7.6 ± 1.0 ab	7.1 ± 0.9 a	15.5 ± 2.3 a	14.4 ± 1.8 ab	61.7 ± 2.8 a	63.6 ± 1.8 a
GT 6	5.8 ± 0.7 b	7.0 ± 0.8 a	13.8 ± 1.8 a	17.8 ± 2.1 a	63.2 ± 2.7 a	69.2 ± 1.5 a*
GT 7	11.0 ± 1.3 a	8.2 ± 1.1 a	18.9 ± 2.7 a	14.8 ± 2.5 ab	57.1 ± 2.4 a	54.0 ± 2.9 c
GT 8	8.5 ± 0.9 ab	8.1 ± 1.0 a	16.8 ± 2.3 a	15.7 ± 2.6 ab	63.5 ± 1.7 a	61.7 ± 1.9 ab

† In each column, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test; (zero insect pressure: df = 7, 7, 7; F = 2.55 for vegetative; 1.28 for reproductive; and 1.02 for reproductive as % of total); (low insect pressure: df = 7, 7, 7; F = 1.53 for vegetative; 2.00 for reproductive; and 3.90 for reproductive as % of total).
*Significant difference ($P \leq 0.05$) between zero and low insect pressure (Student's paired t-test; df = 3).

| | Mean Total Cage Plant Weight (± SE) | | | | | |
| Line | Vegetative | | Reproductive | | Reproductive as % of total | |
	zero†	low	zero	low	zero	low
Westar	193.3 ± 91.6 a	165.0 ± 17.8 b	293.0 ± 123.6 a	230.6 ± 67.1 c	60.3 ± 4.7 ab	57.5 ± 7.9 b
GFP	199.1 ± 49.8 a	168.4 ± 36.6 b	371.6 ± 20.4 a	297.5 ± 23.8 bc*	65.5 ± 5.7 ab	64.1 ± 3.7 ab
GT 1	216.6 ± 29.9 a	214.1 ± 52.4 ab	372.3 ± 78.4 a	404.0 ± 46.7 abc	62.9 ± 2.9 ab	65.5 ± 6.5 ab
GT 4	214.5 ± 18.4 a	188.7 ± 30.8 ab	440.4 ± 69.1 a	345.7 ± 170.1 abc	67.1 ± 2.8 a	61.8 ± 11.0 ab
GT 5	236.2 ± 29.6 a	255.9 ± 16.0 a	478.6 ± 93.6 a	494.3 ± 118.0 ab	66.6 ± 4.3 ab	65.4 ± 4.0 ab
GT 6	206.1 ± 32.1 a	201.1 ± 71.0 ab	434.5 ± 60.4 a	544.5 ± 49.4 a	67.8 ± 3.6 a	73.7 ± 6.4 a
GT 7	231.8 ± 54.4 a	215.0 ± 35.5 ab	367.0 ± 151.6 a	318.0 ± 166.9 bc	59.5 ± 8.1 b	56.2 ± 12.3 b
GT 8	203.9 ± 27.2 a	231.6 ± 29.8 ab	388.5 ± 97.1 a	397.2 ± 118.4 abc	65.1 ± 3.9 ab	62.3 ± 6.3 ab

† In each column, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test: (zero insect pressure: df = 7, 7, 7; F = 0.57 for vegetative; 1.52 for reproductive; and 2.22 for reproductive as % of total); (high insect pressure: df = 7, 7, 7; F = 1.94 for vegetative; 2.94 for reproductive; and 1.95 for reproductive as % of total).
*Significant difference ($P \leq 0.05$) between zero and low insect pressure (Student's paired t-test; df = 3).

Table 3. Mean plant weight (± SE) (vegetative and reproductive components, and reproductive component expressed as proportion of total plant weight) of *Brassica napus* plants transformed with GFP and *Bt* (GT *B. napus* lines), and non-GT *B. napus* cv Westar, and of non-GT *Brassica rapa* populations from Quebec (QB1 and QB2) and California (CA), and the second back-cross of GFP and *Bt* transformed *B. rapa* with parental lines (CA-BC$_2$, QB1-BC$_2$, QB2-BC$_2$) grown under zero or low insect pressure from diamondback moth, *Plutella xylostella*, in Ottawa in 2001. Values shown are mean individual weight per plant for 10 plants harvested separately per replicate, and mean total cage plant weight based on 40 plants per cage.

	Mean Individual Plant Weight (± SE)					
Line	**Vegetative**		**Reproductive**		**Reproductive as % of total**	
	zero†	**low**	**zero**	**low**	**zero**	**low**
Westar	7.30 ± 0.81 a	7.66 ± 0.69 a	14.78 ± 1.71 a	15.38 ± 1.56 ab	65.65 ± 1.66 a	65.21 ± 0.87 a
GT 1	7.42 ± 0.90 a	5.90 ± 0.59 b	16.89 ± 1.82 a	13.57 ± 1.53 b	69.45 ± 1.44 a	67.23 ± 1.13 a
GT 7	8.08 ± 0.66 a	8.52 ± 0.68 a	16.12 ± 1.34 a	17.46 ± 1.44 a	65.90 ± 0.81 a	66.88 ± 0.65 a
CA	6.46 ± 0.77 a	5.64 ± 0.53 a	13.75 ± 1.27 a	11.21 ± 1.20 a	68.01 ± 1.63 ab	64.72 ± 1.48 a
CA-BC$_2$	5.36 ± 0.80 ab	5.78 ± 0.77 a	11.46 ± 2.10 a	11.45 ± 1.50 a	63.60 ± 2.85 b	63.93 ± 2.08 a
QB1	4.95 ± 0.72 ab	5.81 ± 0.61 a	10.45 ± 1.45 a	12.35 ± 1.80 a	67.39 ± 1.60 ab	64.77 ± 1.76 a
QB1-BC$_2$	3.92 ± 0.48 b	4.45 ± 0.51 a	9.64 ± 1.17 a	9.56 ± 1.44 a	71.32 ± 1.94 a	64.53 ± 2.12 a**
QB2	4.90 ± 0.59 ab	4.91 ± 0.52 a	10.64 ± 1.60 a	10.88 ± 1.43 a	66.14 ± 1.62 ab	67.11 ± 1.69 a
QB2-BC$_2$	8.26 ± 3.20 ab	4.53 ± 0.92 a	14.93 ± 4.48 a	12.31 ± 3.46 a	64.28 ± 2.83 ab	69.37 ± 2.55 a

† In each column, within each species, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test: (*B. napus*, zero insect pressure: df = 2, 2, 2; F = 0.97 for vegetative; 1.06 for reproductive; and 1.58 for reproductive as % of total); (*B. napus*, low insect pressure: df = 2, 2, 2; F = 4.93 for vegetative; 2.63 for reproductive; and 0.92 for reproductive as % of total); (*B. rapa*, zero insect pressure: df = 5, 5, 5; F = 1.42 for vegetative; 1.04 for reproductive; and 2.62 for reproductive as % of total); (*B. rapa*, low insect pressure: df = 5, 5, 5; F = 0.93 for vegetative; 0.85 for reproductive; and 0.83 for reproductive as % of total).
**Significant difference ($P \le 0.01$) between zero and low insect pressure (Student's paired t-test; df = 3).

	Mean Total Cage Plant Weight (± SE)					
Line	**Vegetative**		**Reproductive**		**Reproductive as % of total**	
	zero †	**low**	**zero**	**low**	**zero**	**low**
Westar	205.1 ± 40.3 a	208.0 ± 21.6 a	482.7 ± 53.5 a	430.1 ± 47.7 b	70.2 ± 4.6 a	67.4 ± 0.2 a
GT 1	196.6 ± 38.0 a	207.2 ± 45.5 a	511.5 ± 56.4 a	514.1 ± 31.7 a	72.3 ± 3.9 a	71.6 ± 3.6 a
GT 7	249.7 ± 36.8 a	250.0 ± 9.5 a	554.5 ± 35.2 a	562.6 ± 39.0 a	69.0 ± 2.5 a	69.2 ± 1.4 a
CA	204.6 ± 15.9 a	201.5 ± 49.8 a	524.2 ± 54.3 a	397.2 ± 118.1 a	71.8 ± 3.7 a	66.1 ± 4.0 a
CA-BC$_2$	170.1 ± 23.4 ab	190.2 ± 23.1 ab	415.9 ± 5.3 ab	445.7 ± 34.8 a	71.0 ± 2.9 a	70.1 ± 2.9 a
QB1	151.7 ± 16.7 ab	153.5 ± 8.9 abc	399.9 ± 61.8 b	361.4 ± 76.5 a	72.4 ± 0.9 a	69.7 ± 5.1 a
QB1-BC$_2$	136.9 ± 24.6 b	131.5 ± 14.2 c	405.3 ± 49.9 ab	341.0 ± 66.4 a	74.8 ± 2.7 a	71.6 ± 6.5 a
QB2	133.6 ± 24.6 b	146.0 ± 50.0 bc	346.8 ± 81.6 b	362.2 ± 59.1 a	72.0 ± 3.1 a	71.3 ± 9.2 a
QB2-BC$_2$	173.4 ± 70.4 ab	137.2 ± 20.0 bc	405.7 ± 86.4 b	388.1 ± 42.1 a	69.9 ± 13.0 a	73.9 ± 0.7 a

† In each column, within each species, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test; (*B. napus*, zero insect pressure: df = 2, 2, 2; F = 3.33 for vegetative; 1.48 for reproductive; and 0.99 for reproductive as % of total); (*B. napus*, high insect pressure: df = 2, 2, 2; F = 2.13 for vegetative; 10.40 for reproductive; and 2.28 for reproductive as % of total). (*B. rapa*, zero insect pressure: df = 5, 5, 5; F = 2.44 for vegetative; 3.17 for reproductive; and 0.46 for reproductive as % of total); (*B. rapa*, high insect pressure: df = 5, 5, 5; F = 3.18 for vegetative; 1.40 for reproductive; and 0.47 for reproductive as % of total).

Table 4. Mean plant weight (± SE) for vegetative and reproductive components, and reproductive component expressed as proportion of total plant weight of *Brassica napus* plants transformed with GFP and *Bt* (GT *B. napus* lines), and non-GT *B. napus* cv Westar grown under zero or high insect pressure from diamondback moth, *Plutella xylostella* in Saskatoon in 2002. Values shown are mean individual weight per plant for 10 plants harvested separately per replicate, and mean total cage plant weight based on 40 plants per cage.

	Mean Individual Plant Weight (± SE)					
Line	Vegetative		Reproductive		Reproductive as % of total	
	zero †	high	zero	high	zero	high
Westar	8.2 ± 0.9 a	5.6 ± 0.6 a*	10.7 ± 1.2 a	5.6 ± 0.9 b**	56.5 ± 1.0 b	44.2 ± 2.3 b
GFP	7.9 ± 1.2 a	7.7 ± 1.1 a	12.0 ± 1.6 a	4.6 ± 0.9 b**	60.6 ± 1.0 ab	33.0 ± 3.0 c**
GT 6	7.2 ± 0.4 a	8.9 ± 0.7 a*	14.2 ± 0.7 a	15.5 ± 0.9 a	66.4 ± 0.7 a	64.2 ± 0.8 a*

† In each column, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test; (zero insect pressure: df = 2, 2, 2; F = 0.92 for vegetative; 2.55 for reproductive; and 4.04 for reproductive as % of total); (high insect pressure: df = 2, 2, 2; F = 1.43 for vegetative; 50.06 for reproductive; and 94.44 for reproductive as % of total).
*Significant difference ($P \leq 0.05$) between zero and low insect pressure (Student's paired t-test; df = 3).
**Significant difference ($P \leq 0.01$) between zero and low insect pressure (Student's paired t-test; df = 3).

	Mean Total Cage Plant Weight (± SE)					
Line	Vegetative		Reproductive		Reproductive as % of total	
	zero †	high	zero	high	zero	high
Westar	247.5 ± 35.1 a	211.7 ± 41.0 a	342.2 ± 87.0 b	207.4 ± 17.7 b	57.6 ± 5.3 a	49.7 ± 3.5 a**
GFP	263.7 ± 32.3 a	225.6 ± 51.3 a	391.5 ± 53.4 b	140.6 ± 92.4 c*	59.7 ± 4.5 a	33.6 ± 20.3 b
GT 6	285.5 ± 72.8 a	288.8 ± 90.3 a	498.7 ± 67.9 a	507.8 ± 116.7 a	72.9 ± 40.1 a	63.8 ± 5.4 a

† In each column, means with the same letter are not significantly ($P > 0.05$) different using the Waller-Duncan t-test; (zero insect pressure: df = 2, 2,2; F = 0.63 for vegetative; 13.56 for reproductive; and 1.10 for reproductive as % of total); (high insect pressure: df = 2, 2,2; F = 3.56 for vegetative; 21.32 for reproductive; and 11.24 for reproductive as % of total).
*Significant difference ($P \leq 0.05$) between zero and low insect pressure (Student's paired t-test; df = 3).
**Significant difference ($P \leq 0.01$) between zero and low insect pressure (Student's paired t-test; df = 3).

DISCUSSION

Transgenic *B. napus* plants that produce *Bt* Cry1Ac protein are lethal to individuals of diamondback moth populations collected from eastern and western Canada. Previous studies (Ramachandran et al., 1998a, 1998b, 1998c; Stewart et al., 1996, 1997) also demonstrated that transgenic *B. napus* producing Cry1Ac protein are lethal to diamondback moth. Our results indicated that when the *Bt* gene was transferred to wild *B. rapa* populations, production of the Cry1Ac protein occurred and resistance to diamondback moth was conferred. Although a plant fitness advantage was not observed for either species under low insect pressure, under high insect pressure the trait is likely to provide a competitive advantage to the *Bt*-producing *B. napus* crop, to its weedy volunteers and

to weedy *B. rapa* populations (although not specifically tested in this study), increasing the need for improved weed management. The presence of the *Bt* trait in wild crucifer populations and persistence of the *Bt* trait in volunteer or feral *B. napus* could also have a potential ecological impact on the insect populations – such as increasing exposure of diamondback moth populations to the *Bt* toxin and subsequent selection of resistant individuals, or it may even result in lower diamondback moth numbers due to the elimination of an alternate food source.

The presence of *Bt* on or in *B. napus* plants does not affect selection of plants by diamondback moth for oviposition (Ramachandran et al., 1998c). Therefore, in nature diamondback moth larvae that hatch from eggs (neonates) laid on *B. napus* are the life stage first exposed

to *Bt* toxins from *Brassica* spp. genetically modified to express *Bt* genes or even plants treated with commercial *Btk* (depending on application time). Neonates mine into leaves to feed and do not move between plants until after the first molt (Ramachandran et al., 1998b). Because the *Bt* gene is expressed constitutively in transgenic plants all larvae mining into leaves are exposed to lethal doses and, unless resistant, are killed (Ramachandran et al., 1998c). In contrast, diamondback larvae mining into leaves surface-sprayed with a commercial formulation of *Btk* are exposed to the *Bt* toxin only during the period when they chew through the epidermal layer. Any larvae that do not consume a lethal dose before reaching the middle tissue layers will survive at least until they molt, emerge from the mine and recommence feeding on surface tissues. If the lethal concentration of *Bt* toxin sprayed on the leaf surface was washed away or decayed during the time the diamondback moth larvae were in the leaf mines these individuals would have escaped exposure to a lethal dose. However, our results showed that neonate diamondback moth exposed to the LD_{95} concentration of commercial *Btk* applied to the leaf surface in fact consumed a lethal dose before mining into the leaf.

Resistance of diamondback moth to *Btk* formulations has been widely reported (Ferré and van Rie, 2002) and is an inherited recessive or partially recessive trait (Tabashnik, 1994). Laboratory selection experiments for *Bt* resistance typically expose selected life stages (*e.g.* 3rd or 4th instars) rather than continuous exposure for the entire life cycle (González-Cabrera et al., 2001; Liu et al., 2001; Sayyed et al., 2000). Method of *B. thuringiensis* toxin delivery has impact on susceptibility of diamondback moth neonates and early instar larvae. Based on response of diamondback moth larvae to diet incorporated assays, Asano et al. (1993) suggested that neonates may have a higher tolerance to *B. thuringiensis*. Similarly, Liu et al. (1995) found that 3rd instar diamondback moth from both *Bt*-susceptible and *Bt*-resistant colonies were more susceptible than neonates to leaf disks treated with Dipel 2X HD-1 strain of *Btk*, Abbott). In contrast, our results agree with Roush (1994), who found that survival in F_1 progeny derived from a cross between resistant and susceptible parents was lower for neonates than for late instar diamondback moth fed transgenic broccoli expressing the Cry1Ac protein. In our study, transgenic plants containing *Bt* genes were generally more effective at killing neonates than leaves surface-treated with toxin (LD_{95} rate).

Older diamondback moth larvae showed greater variability in mortality and consumption of transgenic *Bt*-producing *Brassica* plants. This may have been due to

non-uniform physiological condition of the larvae at the start of the experiment. Although 4th instar diamondback moth are ca. the same size this final larval stage ranges from a voracious feeding state to a non-feeding state as larvae are preparing to pupate. Thus, use of neonates hatching from eggs placed on test material will provide the most uniform results in bioassays compared with those that use 3rd or 4th instars.

Our original field cage experimental design intended to use high diamondback moth pressure similar to that used by Stewart et al. (1997) and Ramachandran et al. (1998a). These authors infested cages by applying neonates hatched from eggs obtained from a commercial source at rates of >3000 per plant. Because we studied wild populations collected in Canada a commercial source was not an option. In 2001, despite scale-up of cultures, too few diamondback moths were produced and abnormally warm and dry conditions caused the plants to bolt sooner than expected. Although all available adults and larvae were added to cages, low densities (1 larva per leaf) resulted. In 2002, diamondback moths were added to cages well before plants bolted enabling the populations to increase to high densities (>10 larvae per leaf). These circumstances provided an opportunity to observe effects of *Bt*-producing *B. napus* on plant fitness at low and high insect pressure.

The field cage experiments confirmed that leaf damage by diamondback moth larvae is significantly less on GT *B. napus* or GT *B. rapa* × *B. napus* hybrids than non-transgenic *B. napus* or *B. rapa*. The results suggest that when diamondback moth populations are low, no measurable plant fitness advantage of the *Bt* trait was observed in *B. napus* or *B. rapa*. The reproductive plant weight data suggest increased fitness of GT *B. napus* is only evident when diamondback moth population densities are high. As Ramachandran et al. (1998b) showed, pure stands of GT *B. napus* reduce diamondback moth numbers or density while mixed stands of GT and non-GT *B. napus* result in limited pest reduction due to the ready movement of diamondback moth larvae between plants. Our laboratory results further suggest that mature diamondback moth larvae can survive the ingestion of non-lethal doses (*i.e.* up to 12.3 mm^2 of leaf material) of GT *B. napus* and GT *B. rapa*. Establishment of the *Bt* transgene in wild *B. rapa* populations may also increase its competitive advantage under high insect pressure. Unfortunately, natural densities of diamondback moth on *B. rapa* are not known. Fitness, defined as the reproductive success or the proportion of genes an individual leaves in the gene pool of a population, is the major factor affecting the establishment

and spread of an "escaped" transgene (van Raamsdonk and Schouten, 1997; Warwick et al., 1999). Seed production, one of the most common measures of plant fitness, was estimated in the present study by reproductive weight due to the regulatory restrictions imposed in our confined field trial. The positive selective advantage of the *Bt* transgene is not restricted to agricultural habitats, as is the case for a herbicide resistance transgene. There does not appear to be a cost to the *Bt* trait in GT *B. napus* or GT *B. rapa*, and the *Bt* transgene would, therefore, not be selected against, and could persist in populations in the absence of insect pressure. The interspecific hybridization process itself may also affect the fitness of plants. When F_1 hybrids between weedy *B. rapa* and *B. napus* were grown with their parents under field conditions, the hybrids were significantly more fit than *B. rapa* (Hauser et al., 1998a). Both backcrosses and F_2 hybrids, however, had somewhat reduced fitness relative to the original wild parent (Hauser et al., 1998b).

In Canada, diamondback moth tends to immigrate from the southern USA and Mexico, and population numbers are highly variable from year to year, thus limiting the economic benefit of *Bt*-producing canola varieties. However, if diamondback moth was to regularly establish in canola growing regions, either as a result of adaptation to a cooler Canadian climate or a shift in environmental conditions that would be more conducive to winter survival, the economic benefit of *Bt*-producing canola varieties would increase. Commercialization of canola varieties producing the *Bt* Cry1Ac protein is not recommended, as they are likely to fail rapidly given the current extent of resistant populations of diamondback moth. If *Bt*-producing canola was introduced, resistance management would become increasingly important as would the role of *Bt*-producing wild populations of *B. rapa*.

MATERIALS AND METHODS

Plant material

Brassica napus

The transformed *Brassica napus* cv Westar lines used were developed by Halfhill et al. (2001) and included two GFP (green fluorescent protein) constructs, *mGFP5er* (GFP only) and pSAM 12 (GFP linked to a synthetic *Bacillus thuringiensis cry1Ac* gene; nine GT lines). Nine lines of <u>G</u>FP-B<u>T</u> (GT) and two lines of GFP *B. napus* were selfed and progeny screened to obtain homozygous lines. *Brassica napus* cv Westar was used as a control.

Brassica rapa

Nine GT and two GFP *B. napus* lines were hybridized with three wild accessions of *B. rapa*, one population, CA, from Irvine, California, USA (33°40'N 117°49'W) and two populations from Quebec, Canada, QB1 (Milby, 45°19'N 71°49'W) and QB2 (Waterville, 45°16'N 71°54'W) (Halfhill et al., 2001). F_1 hybrids and BC_1 progeny were screened and individuals showing the presence of the GFP-*Bt* transgenes were backcrossed onto the appropriate *B. rapa* parent (CA or QB1 or QB2) to produce BC_1 and BC_2 generations, respectively. *B. rapa* hybrid plants were only used in the Ontario field trial (since these lines involved weedy populations from Quebec and California, it was not permissible to import them to Saskatchewan, where *B. rapa* is grown as a crop).

Insect populations

Diamondback moth cultures were established from larvae collected in Saskatchewan in 1998 (western population) and in Ontario in 1999 (eastern population). Cultures were maintained either on *B. napus* (*B. napus* cv Excel) (eastern and western) or a wheat germ-casein artificial diet (Shelton et al., 1991) (western). Insects were reared at 22 ± 2 °C or 27 ± 2 °C (eastern and western, respectively), 50 ± 3.5% RH, and a 16:8 light:dark photoperiod during all life stages.

Laboratory insect bioassays

Preliminary bioassays were performed to determine the LD_{50} and LD_{95} values of Safer's BTK™ Biological Insecticide (*Btk*) [Safer Ltd., Box 186, Scarborough Ontario (*Bacillus thuringiensis* subspecies *kurstaki*; potency 10 600 IU.mg^{-1} equivalent to 12.6 billion international units per L)] against diamondback moth larvae (data not shown). Experimental bioassays included a set of control treatments: *B. napus* leaf disks (12.5 mm diameter) that were either untreated ('dry'), or dipped in water ('wet') or in the LD_{50}, LD_{95}, or recommended field rate of *Btk*, allowed to air dry for 15 min, and placed on 1.2% agar. Test treatments included leaf disks from one GFP *B. napus* line, nine GT *B. napus* lines, and GT *B. rapa* F_1's from Quebec (QB1, QB2) and BC_2 lines from California (CA) and Quebec (QB1, QB2). Controls used were: cv Westar for *B. napus* bioassays and CA or QB1 or QB2 lines for *B. rapa* bioassays (with *B. napus* cv Westar included as cross-reference between the two species).

For each treatment, arenas (NuTrend 12- or 24-well plates) were filled with 1mL aliquots of agar using a repeater pipette (BrandTech Scientific HandyStep®). One leaf disk was placed in each well and eggs (from which neonates hatched) or 4th instars were placed individually on leaf disks. The plates were sealed with Mylar, aerated by pin-hole punctures in the Mylar, and maintained at 22 ± 2 °C, $50 \pm 2\%$ RH, and 16:8 h light:dark. Larval condition (active, lethargic, near death, pupated) and mortality were recorded every 1–3 days, as well as leaf damage (0–10 damage scale: 0 = no damage, 10 = 100% damage). Cumulative damage was an estimate of total leaf area consumed over the test period. Leaf disks were changed daily as required. Insect mortality, total leaf area consumed and final stage reached were compiled for observation periods 5–7, 10–14 (neonate and 4th instar), and 18–21 (neonate only) days after start of the experiments.

Field plant fitness trials

Field experiments were conducted at the Central Experimental Farm, Ottawa, Ontario (eastern location, 45°25'N 75°43'W) in 2001 and the Saskatoon Research Centre farm, Saskatoon, Saskatchewan (western location, 52°07'N 106°38'W) in 2001 and 2002. The experiments were performed in cages to ensure that numbers and species of insect herbivores could be controlled. At each location the field was divided into four blocks and a main plot-subplot design was used with treatments randomly placed within each block. At the eastern location, no herbicides or fertilizer treatments were applied to the plot area. At the western location, plots were treated with pre-emergent Edge granular (ethafluralin) (Dow Agro-Sciences) at 3.67 kg.ha^{-1} for weed control. The land was treated the previous fall with N, P, K, S fertilizer 65-35-10-15 at 41.99 kg.ha^{-1} in 2001 and 45-40-10-15 at 35.86 kg.ha^{-1} in 2002.

Eastern location (2001)

In each of the four blocks, nine 3m × 3m plots were established, and two 1 m^2 cage-areas within each plot were either seeded 21 May by hand at 40 seeds/m^2 with non-transgenic *B. napus* cv Westar (control) or GT *B. napus* [lines GT-1, GT-7], or planted 6 June with 3-4 leaf stage seedlings of non-transgenic *B. rapa* CA, QB1, QB2 (controls), or GT *B. rapa* BC$_2$ lines (CA-BC$_2$, QB1-BC$_2$ or QB2-BC$_2$). There were a total of 72 cages. The area outside the cage-areas was unseeded. Two fine-mesh (0.79 mm mesh) cages (1m^2 × 1.5m high) were set up in

all plots immediately after seedling emergence in the seeded plots. All *B. rapa* seedlings, grown in the greenhouse, were screened for GFP expression using a hand-held UV lamp. Plants containing GFP-*Bt* were transplanted when plants in the seeded plots reached the 3-leaf stage and immediately caged. Plants were removed and/or added to ensure a density of 40 plants/m^2 in each cage. After transplants had established, eastern diamondback moth were added to one of the two cages in each plot (treatment designated: low insect pressure with 60 adults and 60 larvae added per cage between 21 June and 21 July). The second cage remained empty (zero insect pressure). At flowering (13 July), leaf-cutter bees [*Megachile rotundata* (Fab.)] were added to cages (3 gm per cage) containing *B. rapa*, a self-incompatible species, to ensure pollination. Ten plants in each cage were randomly selected and rated for leaf damage. Leaf damage was rated on a 0–10 damage scale: 0 = no damage, 10 = 100% damage. Very small feeding holes were counted as a score of 0.01 (*i.e.* 0.1% eaten). Ratings were performed by the same evaluators twice during the season: before introduction of diamondback moth and when plants were beginning to form seed pods.

At the end of the experiment, plants were removed before pods ripened to prevent any GT seed from escaping [as required by Confined Field Trial permit (Canadian Food Inspection Agency, 2002)]. At harvest plant counts were taken in each cage, the ten plants that had been previously rated for damage were removed individually by cutting stems off at ground level, bagged separately into two bags: reproductive component (green pods, including upper stem branches with pods) and vegetative component (stem, remaining leaves). The rest of the plants from each cage (to a total of 30 plants) were bulked into reproductive and vegetative components. All plant material in paper bags was dried and weights recorded.

Western location (2001 and 2002)

In each of four blocks, eight 3m × 3m plots were established, and two 1 m^2 cage-areas within each plot were seeded as follows: one plot of non-transgenic *B. napus* cv Westar (control), one plot of GFP *B. napus* (control), and six plots of GT *B. napus* (in 2001: GT-1, GT-4, GT-5, GT-6, GT-7, GT-8; 2002 only GT-6). All 1 m^2 cage-areas were seeded (22 May 2001; 31 May 2002) by hand at 40 seeds/m^2. The area outside the cage-areas was unseeded. Cages were installed in each plot after seeding between seedling to rosette stage (2001) or before seedling emergence (2002).

In each plot, western diamondback moth were added to one of the two cages [in 2001, 150 larvae per cage between 11 July to 2 August (low insect pressure); in 2002, 72 larvae and 12 adults per cage between 26 June to 14 August (high insect pressure)]. The second cage remained empty (zero insect pressure). Leaf damage ratings (10 plants each) in control treatment cages and in four GT *B. napus* cages were taken twice during the season (2001) and/or when plants were harvested (2001, 2002). Plants in cages were harvested and processed in the same manner as for the eastern location.

Statistical analyses

For the insect bioassays, consumption data were LOG (x + 1.0) transformed to normalize the data. Analysis of variance was then performed using the general linear models (Proc GLM), and differences between means were determined using the Waller-Duncan K-ratio t-test ($P = 0.05$) (SAS Institute Inc., 1989).

For the field trials, plant weight data were LOG (x + 2.0) transformed and leaf damage data were arcsin (x) transformed prior to analysis to normalize the data. The transformed data were analyzed using the general linear models (Proc GLM), and differences between means were tested for significance using the Waller-Duncan K-ratio t-test ($P = 0.05$) (SAS Institute Inc., 1989). Differences in mean weight for each treatment under zero and either low or high insect pressure were derived from the Student's paired t-test. *B. napus* and *B. rapa* were analysed separately at the eastern location in 2001.

To determine if differences occurred between eastern and western diamondback moth populations, a series of paired bioassays were conducted wherein the same leaf material was fed to each population. Leaf material harvested in Ottawa was transported to Saskatoon overnight for bioassays the following day. For both 4th instars ($n = 3$ paired bioassays) and neonates ($n = 4$ paired bioassays), there were significant differences between the two populations for mortality and total leaf consumption (GLM ANOVA, SAS). As a result, bioassays for eastern and western diamondback moth populations were analyzed separately.

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