

Increased fitness of transgenic insecticidal rapeseed under insect selection pressure

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Abstract

Rapeseed *Brassica napus* L. transgenic for a *Bacillus thuringiensis* (Bt) transgene was developed and was shown to be insecticidal towards certain caterpillars including the diamondback moth *Plutella xylostella* L. and the corn earworm *Helicoverpa zea* Boddie. To simulate an escape of the transgenics from cultivation, a field experiment was performed in which transgenic and nontransgenic rapeseed plants were planted in natural vegetation and cultivated plots and subjected to various selection pressures in the form of herbivory from insects. Only two plants, both transgenic, survived the winter to reproduce in the natural-vegetation plots which were dominated by grasses such as crabgrass. However, in plots that were initially cultivated then allowed to naturalize, medium to high levels of defoliation decreased survivorship of nontransgenic plants relative to Bt-transgenic plants and increased differential reproduction in favour of Bt plants. Thus, where suitable habitat is readily available, there is a likelihood of enhanced ecological risk associated with the release of certain transgene/crop combinations such as insecticidal rapeseed. This is the first report of a field study demonstrating the effect of a fitness-increasing transgene in plants.

Keywords: *Brassica napus*, *Bacillus thuringiensis*, canola, gene flow, ecological risk, GMO, *Plutella xylostella*, *Helicoverpa zea*, population replacement

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Introduction

Rapeseed (canola) *Brassica napus* L. has been successfully grown commercially in the south-eastern USA since 1988, where it has proven to be an economically viable alternative to winter production of soft red winter wheat *Triticum aestivum* L. em Thell. Since the first commercial plantings of genetically modified rapeseed were made in Georgia in 1994, genetically modified plants are becoming more widely commercialized and released into the environment (Dale *et al.* 1993; Sawahel 1994; Rogers & Parkes 1995). Several studies have addressed various issues regarding food safety and the ecological risks of releasing transgenic plants into the environment (Kareiva *et al.* 1994; Kok *et al.*

1994; Regal 1994; Purrington & Bergelson 1995). Most often, the risks of transgene spread and introgression have been studied, while the ecological effects of the transgenes themselves have not been widely addressed (Crawley *et al.* 1993; Linder & Schmitt 1994; Mikkelsen *et al.* 1996). While neutral transgenes may have minimal ecological effects to their hosts and ecological systems (Crawley *et al.* 1993), a transgene conferring increased fitness may have significant effects (Kareiva *et al.* 1994; Linder & Schmitt 1995). Fitness-conferring transgenes may have their greatest effects when they are in a host that may persist outside of cultivation, and/or may be spread from an agricultural host to a weedy relative. It is conceivable that a transgenic crop plant may become weedy or related weeds could become weedier.

To assess a possibly problematic transgenic crop scenario in which a gene may confer fitness, we transformed rapeseed, a crop produced for oilseed, with a synthetic

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Bacillus thuringiensis crystal endotoxin transgene (*Bt cryIAc*) (Stewart *et al.* 1996), and performed an experiment to simulate a transgenic crop escape. We deployed two cultivars transformed and nontransformed, in a tented field study in which herbivorous insect availability was manipulated. Two susceptible caterpillars, the diamond-back moth (DBM) *Plutella xylostella* L. and the corn earworm (CEW) *Helicoverpa zea* Boddie were applied separately and in combination to simulate specialist (DBM) and generalist (CEW) herbivory on the rapeseed. Several fitness-related traits were compared between transgenics and nontransgenics to assess whether populations of transgenic plants may establish themselves outside of cultivation preferentially to nontransgenic plants. We tested the null hypothesis that there would be no fitness difference in transgenic vs. nontransgenic plants in vegetated and cultivated plots.

Materials and methods

Field site

The field experiment was performed on the University of Georgia Horticulture Farm in Watkinsville, GA, which is located in the Piedmont physiographical region. The field, which had been out of cultivation for 10–15 years, had moderately low fertility and over 90% cover of grasses such as *Agrostis* spp., *Andropogon* spp., *Digitaria* spp., *Paspalum* spp., *Cynodon dactylon* and *Hordeum pusillum*.

Experimental design and statistical analyses

A randomized complete-block, split-plot design was used to determine the effects of vegetation type and insect defoliation treatments on rapeseed fitness (*Bt*-transgenic vs. nontransgenic). There were six blocks, each split with cultivation treatments and insect treatments, so every block contained two different cultivation treatments and each cultivation treatment contained five insect treatments. Each insect treatment (plot) contained all four rapeseed genotypes. In summary, the experiment contained five plots nested within each of two cultivation treatments contained within each of six blocks, resulting in 60 plots in the experiment. In all cases, the response variable data are represented per treatment-row of plant genotype. A nested analysis of variance (ANOVA) was performed to determine if the insect treatments had an effect on plant establishment, the number of surviving plants, defoliation, and damage rating (transgenic vs. nontransgenic) (SAS Institute 1990). If significant differences were found Tukeys HSD multiple comparisons test were performed to locate the differences (SAS Institute 1990). In addition, a single degree of freedom *post hoc* contrast was performed to determine if the insect treatments had an effect on seed

production of plants. In all cases the response variables are reported on a genotype per row basis. Negative correlation between defoliation and seed production was tested using a Spearman correlation procedure (SAS Institute 1990).

There now follows is a more detailed description of the treatments and plant genotypes. One-half the 60 plots were cultivated and one-half were left naturally vegetated. The cultivated plots were roto-tilled and seeds were planted in 1-m-long rows. After the seeds were planted no further cultivation was performed. Seeds in the non-cultivated plots were planted in 1-m-long, \approx 1-cm-wide furrows that were created by lightly disturbing the surface of the soil with a wooden stake. Seeds in both cultivation treatments were lightly covered with soil. All plots (1 m²) contained 50 seeds of each of four lines: two cultivars (Oscar and Westar), transformed (OBT and WBT) and nontransformed (O and W). OBT was a moderately high expressing line (0.05% *Bt CryIAC*), and WBT was a low expressing line (0.005% *Bt CryIAC*) (Stewart *et al.* 1996). Nontransformed lines were the null segregants of the transgenic lines used. Plant line was randomized among rows within plots. Plots were randomized within blocks. Seeds were planted 23 September 1995. Each plot was subjected to one of five treatments: tent, A-frame type 1-m² insect cages that enclosed the plants and vegetation; insecticide, tented treatment in which malathion (6.25 mL containing 56.8% active ingredient and diluted in 83.3 mL of water) was applied at 7-day intervals throughout the growing season to keep plots free of insects; CEW, tented treatment in which CEW neonates were applied to all plants with a 'bazooka' (Wiseman, Davis & Campbell 1980) at a rate of 800 larvae per plot row; DBM, tented treatment in which DBM neonates were applied to all plants with a bazooka at a rate of 3150 larvae per plant; CEW + DBM, tented treatment in which both insects were applied together at one-half the above rates. CEW eggs were obtained from the USDA-ARS Insect and Population Management Research Laboratory (Tifton, GA, USA). DBM eggs were obtained from Abbott Laboratories (Chemical and Agricultural Products Division, Chicago, IL, USA). The eggs were hatched in the laboratory and 2–4-h-old neonate larvae were mixed with corn grits prior to application. The insects were applied 30 days after seeds germinated. Insect survivorship and damage data were taken 15, 40 and 150 days after insect applications.

Results

Vegetation effects

The naturally vegetated plots, dominated by established grasses, contained only two (of 6000 seeds planted) rapeseed plants (WBT) that survived the winter to reproduce.

Table 1 Responses of insecticidal and nontransgenic rapeseed to various treatments (DBM, diamondback moth; CEW, corn earworm; described in the Materials and methods). Damage rating of plants and defoliation were assessed at 40 days after treatments. The number of plants per row were assessed the next spring, 6 months after treatments. The types of the plants were as follows: O, Oscar; OBT, Oscar transgenic for *Bt cryIAC*; W, Westar; WBT, Westar transgenic for *Bt cryIAC*. Different letters denote significant differences at $P = 0.05$ (Tukeys HSD within columns). For the number of plants, ** denote significant differences within the column ($P = 0.10$)

Treatment	Plant type	Plants (number)	Defoliation (%)	Damage rating*
CEW	O	8.5 ± 4.7	6.3 ± 5.4 b	3.5 ± 2.4 b
	OBT	10.3 ± 5.6	0.50 ± 1.2 b	0 ± 0 c
	W	7.7 ± 4.4	7.3 ± 6.8 b	3.7 ± 2.3 b
	WBT	8.5 ± 3.5	2.3 ± 1.6 b	0.33 ± 0.52 c
DBM	O	4.4 ± 2.9**	54.0 ± 38.9 a	6.0 ± 1.4 a
	OBT	9.0 ± 4.9	0.60 ± 0.55 b	0.20 ± 0.45 c
	W	2.6 ± 1.9**	61.0 ± 35.8 a	6.4 ± 1.3 a
	WBT	9.8 ± 6.5	4.6 ± 1.7 b	1.4 ± 0.89 c
CEW + DBM	O	9.4 ± 4.7	41.0 ± 18.8 a	6.2 ± 0.45 a
	OBT	12.2 ± 6.5	1.6 ± 1.1 b	0.20 ± 0.45 c
	W	8.0 ± 3.1	52.0 ± 13.0 a	6.8 ± 0.45 a
	WBT	8.4 ± 4.5	5.4 ± 8.2 b	0.80 ± 0.84 c
Tent	O	11.5 ± 5.6	0 ± 0 b	0 ± 0 c
	OBT	12.0 ± 5.3	0.83 ± 2.0 b	0.10 ± 0.05 c
	W	9.2 ± 2.4	3.3 ± 5.2 b	0.21 ± 0.12 c
	WBT	11.0 ± 2.5	0.33 ± 0.82 b	0 ± 0 c
Insecticide	O	11.3 ± 3.8	0 ± 0 b	0 ± 0 c
	OBT	13.7 ± 6.9	0 ± 0 b	0 ± 0 c
	W	11.8 ± 8.5	1.17 ± 2.0 b	0.17 ± 0.41 c
	WBT	9.8 ± 4.1	0.67 ± 1.6 b	0 ± 0 c

* 0–7 plant damage rating of a row. 0, Virtually no damage apparent on plants; 1, < 5% plants with some damage; 2, 5–10% of the plants with damage; 3, 11–20% of the plants with damage; 4, 21–30% of the plants with damage; 5, 31–60% of the plants with damage; 6, 61–80% of the plants with damage; 7, > 81% of the plants with damage.

So, although these two plants were transgenic, it seems that the transgene did not confer the ability to allow rapeseed to outcompete the grasses that were already established.

In cultivated plots there were no differences among transgenic and nontransgenic cultivars in establishment after planting, although Oscar (transgenic and nontransgenic) had better establishment than Westar (21.2 vs. 17.5 plants per plot row 30 days after planting, $P < 0.05$) regardless of transgene status. In contrast with noncultivated plots there was no catastrophic survivorship decline after winter (Table 1). Furthermore, there were no apparent morphological differences between transgenic and nontransgenic plants prior to insect treatments.

Insect effects

To assess the effect a single defoliation episode of CEW and DBM, we measured defoliation and damage rating of the plants, and also carried out a census of insects in cultivated plots. There were means of 2.8 CEW, 19.6 DBM, and 17.2 CEW and DBM in each of respective plot 15 days after application. These were all significantly different from zero. We characterize the level of infestation as a single small infestation. There were no appreciable nonapplied

herbivores present in any of the tented treatments other than a negligible number of aphids. There were significant effects in defoliation and damage rating as the result of the DBM and the CEW + DBM treatments (Table 1, Fig. 1). In addition, the damage rating for CEW was significant. It is clear that the *Brassica* specialist, DBM had a prominent defoliation effect on the plants at the end of the autumn growing season. The effect of the CEW infestation was less clear.

It is not unexpected that there would be profound differences in defoliation between transgenics and nontransgenics as the result of a DBM infestation, because the transgenic plants harbour a strongly effective insecticidal transgene. To determine whether defoliation differences translated to differences in reproductive success, we monitored plant survivorship and reproductive effort throughout the following winter and autumn. There were no differences in overwinter survivorship among plants except for within the DBM treatment, where there was a marginally significant affect noted among transgenics and nontransgenics at $P = 0.10$ (Table 1). Because of the variability of apparent insect establishment and damage among DBM plots, strong statistical power was lacking in the ANOVA. Thus, the large amount of defoliation did not effect plant survivorship



Fig. 1 Photographs of a plot where diamondback moths were applied at a rate of 3150 larvae per plot. (A) The plot 1 month after infestation while plants are in vegetative rosettes. (B) The plot (foreground) 6 months after infestation while plants are flowering. The two rows on the left contain *Bt* transgenic plants while the two rightmost rows (arrows) contain nontransgenic plants. Defoliation in this plot was negligible for transgenics and over 95% for nontransgenics.

except in the extremely defoliated plots (Fig. 1). None the less, there were significant effects of defoliation on seed production (Fig. 2). When only the DBM treatment is considered, the nontransgenics suffered various degrees of defoliation (from 5 to 98%) and never produced more than 80 seeds per row (Fig. 2). However, the transgenic plants never suffered more than 5% defoliation and seed production ranged from 0 to over 750 seeds per row (Fig. 2). Using a single degree of freedom contrast, the transgenic plants produced significantly more seeds than nontransgenic plants. When all tented treatments are considered and ratios of nontransgenic to transgenic plants are computed, the same significant (at $P = 0.05$) inverse relationship between defoliation and seed production is

observed using a Spearman correlation (Fig. 2). In this analysis, when the non-transgenic/transgenic seed ratio equals one, then there are equal amounts of seed produced by each type of plant. When this ratio is greater than one, there are more seeds produced by nontransgenics compared with transgenics. The ratio greater than one was observed in only 17% of the plots where insects were applied, but was twice that frequency in plots where insects were not applied. Thus, herbivory by insects was an effective selection agent in favour of transgenic insecticidal plants. Furthermore, effective selection only required low differentials of insect numbers (on nontransgenic vs. transgenic) and a single episode of herbivory.

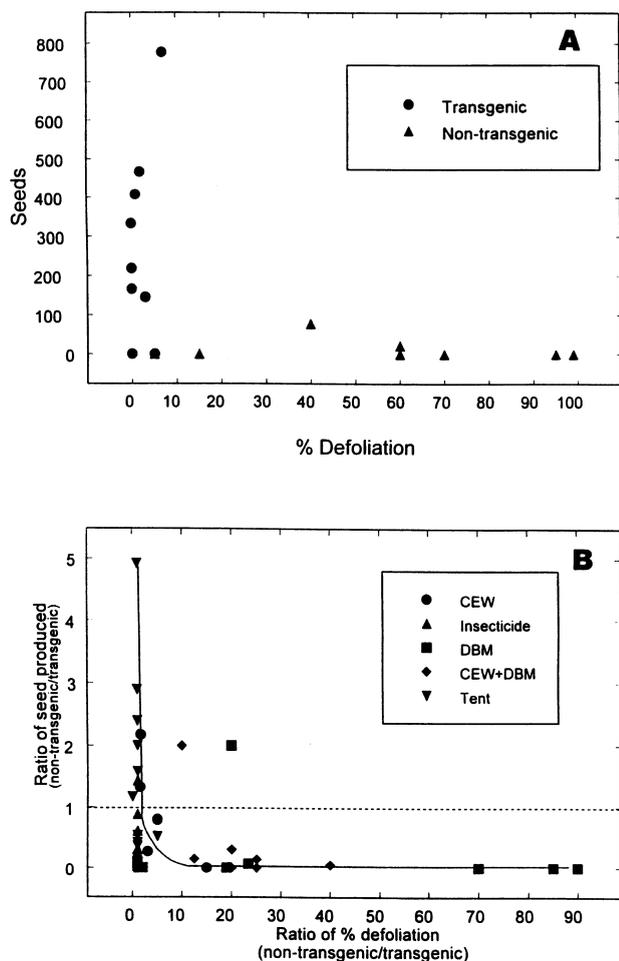


Fig. 2 The relationship of percentage defoliation recorded in November 1995 and the resultant seed production of rapeseed in May 1996. Data from the diamondback moth treatment are displayed (A), and ratios of nontransgenics to *Bt CryIAC* transgenics for percentage defoliation and seed production (B). In (B), each data point represents a plot row of a cultivar non-transgenic/transgenic (e.g. Oscar/*Bt CryIAC* Oscar). The treatments are described in the Materials and methods.

Discussion

Rapeseed is a new crop in the south-eastern USA, California, and other warm temperate areas where it has not been historically grown. It is in these areas that herbivory is expected to have an ever-increasing impact on rapeseed production (Lamb 1989; Buntin & Raymer 1994). Likewise, in these areas *Bt*-transgenic rapeseed may be of agronomic benefit (Stewart *et al.* 1996), hence the rationale for performing transgenic plant survivorship experiments in Georgia.

Because of the low overwinter survivorship of rapeseed in the noncultivated plots, it is unlikely that an insecticidal gene will allow canola-quality rapeseed to become invasive

in undisturbed habitats that receive no regular natural or human perturbations. However, year-to-year variation in neighbour densities and weather could alter survivorship of rapeseed with respect to interspecific competitors. Anecdotal evidence suggests that rapeseed can survive quite easily on roadsides in Georgia, which are planted with turfgrass and are regularly mowed. In and around Griffin, Georgia, where rapeseed breeding has been performed for only 10 years, there are several prominent roadside populations which presumably did not exist prior to breeding. This demonstrates that canola-quality rapeseed can naturalize under certain conditions in Georgia.

This study demonstrates that insecticidal rapeseed could pose an ecological risk upon environmental release. There are three effects that lead us to this conclusion. First, insecticidal rapeseed that highly expresses the *Bt cryIAC* gene is highly resistant to ubiquitous susceptible defoliating lepidopterans such as DBM (Table 1; Stewart *et al.* 1996). Second and most importantly, a single episode of herbivory by diamondback moth decreased the overwinter survivorship of nontransgenic rapeseed (Table 1, Fig. 1). Third, where there was extensive defoliation, there was very low seed production in nontransgenic rapeseed (Fig. 2). Because rapeseed is already a minor weed in areas (Williamson 1992), the ability to strongly resist defoliation may allow it to selectively persist to a greater extent by replacing nontransgenic naturalized populations (Hoffman 1990; Andow 1994; Dale 1994; Schmitt & Linder 1994). As Andow (1994) has pointed out using mathematical modelling, a drastic alteration in a single trait could increase a plant's competitiveness, thereby allowing it to impact natural plant populations and communities. There are several examples of single trait alterations that have impacted plant competitiveness. One of which is chestnut blight (Andow 1994), in which the lack of resistance to a single pathogen reduced American chestnut (*Castanea dentata*) from a dominant tree to an incidental, non-flowering shrub throughout its distribution. It is foreseeable that the introduction of a single profound trait could also cause a mild shift in the weediness potential of rapeseed. As canola-quality rapeseed has been selected for, among other things, low seed dormancy, it will likely not become a persistent weed in undisturbed habitats. On the basis of theoretical and experimental work, Rees & Long (1992) and Linder & Schmitt (1995) argue that it is unlikely for enhanced vegetative or reproductive characters to lead to increased fitness unless a species has a persistent and viable seedbank and that seed germination is enhanced by disturbance. Rapeseed does not fit this profile well.

Although rapeseed itself may not become a weed, fitness-enhancing transgenes may introgress into wild relatives under open pollinating conditions. Jorgensen &

Anderson (1994) report 9–93% hybridization rate for *B. napus* × *B. campestris*. Indeed, recently Mikkelsen *et al.* (1996) also showed high hybridization and introgression frequencies between *B. napus* and *B. campestris*. *Brassica napus* is also sexually compatible with other weedy mustards. In some of the most thorough work to date, Bing (1991) showed that *B. napus* crosses successfully at a high rate with *B. juncea*, and at a much lower rate with *B. nigra*. However, no fertile hybrids were obtained by *B. napus* × *Sinapsis arvensis* (wild mustard). In fact the only fertile hybrids produced between *B. napus* and *S. arvensis* under open fertilization conditions have been when male-sterile *B. napus* was used as the female parent (Lefol, Danielou & Darmency 1996). Scheffler & Dale (1994) have reviewed the compatibility among members of the Brassicaceae. They reported that *Raphanus raphanistrum* and five other weedy relatives are compatible with rapeseed. So, if *Bt* rapeseed were to be widely deployed in nature it is conceivable that transgenic *B. napus* could transfer its insecticidal *Bt* gene into up to nine wild relatives quite rapidly. If one examines the number of additional species that are sexually compatible with *B. campestris*, *B. juncea* and *B. nigra*, the introgression list lengthens to include wild mustard and several other species.

Given the above data and scenarios, and the complex taxonomic relationship in the Brassicaceae, a gene conferring insect resistance will likely become fixed in weedy mustards in ecological time. Insect-resistant wild mustard is certainly not a desirable organism in either agricultural or natural ecosystems (Kareiva, Morris & Jacobi 1994). An effect seen in a small experiment such as this raises concern about large-scale releases into the environment of commercialized transgenic plants. A system for monitoring the effects of fitness related transgenes may be desirable to assay long-term effects (Stewart 1996a,b).

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Neal Stewart is interested in the long-term ecological effects of transgenic plant release and population ecology. Paul Raymer is a canola breeder and agronomist interested in the application of transgenes for cultivar improvement. John All and Suresh Ramachandran are entomologists who are interested in integrated pest management. The contribution of Suresh Ramachandran in the study represents partial fulfilment of his PhD project with transgenic insecticidal rapeseed.
