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## Impact of ecological factors on the initial invasion of *Bt* transgenes into wild populations of birdseed rape (*Brassica rapa*)

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**Abstract** The inevitable escape of transgenic pollen from cultivated fields will lead to the emergence of transgenic crop-wild plant hybrids in natural patches of wild plants. The fate of these hybrids and that of the transgene depend on their ability to compete with their wild relatives. Here we study ecological factors that may enhance the fitness of genetically modified hybrids relative to wild plants for a *Bacillus thuringiensis* (*Bt*) transgene conferring resistance to insects. Mixed stands of wild plants and first-generation hybrids were grown under different conditions of herbivore pressure and density, with *Bt* oilseed rape (*Brassica napus*) as the crop and *B. rapa* as the wild recipient. Biomass and fitness components were measured from plant germination to the germination of their offspring. The frequency of transgenic seedlings in the offspring generation was estimated using the green fluorescent protein marker. The biomass of F<sub>1</sub> *Bt*-transgenic hybrids relative to that of wild-type plants was found to be sensitive to both plant density and herbivore pressure, but herbivore pressure appeared as the major factor enhancing their relative fitnesses. In the absence of herbivore pressure, *Bt* hybrids produced 6.2-fold fewer seeds than their wild neighbors, and *Bt* plant frequency fell from 50% to 16% within a single generation. Under high herbivore pressure, *Bt* hybrids produced 1.4-fold more seeds, and *Bt* plant frequency was 42% in the offspring generation. We conclude that high-density patches of highly damaged

wild plants are the most vulnerable to *Bt*-transgene invasion. They should be monitored early to detect potential transgene spread.

### Introduction

The frequency of spontaneous interspecific hybridization between vascular plant species and the subsequent persistence of hybrid descendants have been widely documented for several decades (Stebbins 1959; Raven 1976; Whitham et al. 1991; Ellstrand et al. 1996). An estimated 70% of all angiosperm species owe their origins to interspecific hybridization (Masterson 1994). Spontaneous hybridization is the rule in some groups of vascular plants (Ellstrand et al. 1996), and this likewise applies to some agricultural crop species, based on evidence for gene flow between them and their wild relatives (Raybould and Gray 1994; Darmency et al. 1998; Ellstrand et al. 1999; Jenczewsky et al. 1999). Since the escape of pollen from cultivated fields is inevitable (Kareiva et al. 1994), this reasoning also applies to transgenic crops, and the presence of introgressed transgenic DNA in wild plant populations has been reported (Quist and Chapela 2001; but see Kaplinsky et al. 2002). It seems increasingly clear that transgenic crop-wild plant hybrids will emerge in natural patches of wild plants following the commercialization of transgenic crops (Ellstrand 2001). Thus, the relevant issue in assessing the risk of spread of transgenes is not hybridization probabilities, but rather the probability of spread and persistence of transgenes into wild plant populations.

Transgenes can spread and persist in wild populations either through the back-crossing of transient transgenic hybrids with wild-type plants or by the stabilization and subsequent increase in the frequency of a transgenic hybrid line. These two events—i.e. successful introgression of wild-type plants or invasion by transgenic hybrids—are respectively considered as a threat to the genetic diversity of wild plants and the biodiversity of natural communities (Kling 1996; Hails 2000; Snow 2002). The

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probabilities of their occurrence depend on the initial persistence of the transgenes in wild populations and, therefore, on the competition of first-generation hybrids with their wild neighbors (Hauser et al. 1998a). Ecological fitness studies are important in this regard, because without detailed examination, risk assessments may overlook the possibility that transgenic hybrids have higher fitnesses than their wild relatives, leading to the invasion of the former (Raybould and Gray 1994; Wolfenbarger and Phifer 2000; Hails 2002).

Enemy-resistant genetically modified hybrids (e.g. insect herbivore-resistant, fungi-resistant or virus-resistant plants) present two features—hybrid genomes and enemy resistance—that may foster their invasiveness by releasing them from genetic and ecological constraints. First, hybridization and concurrent genome restructuring can rapidly generate novel, fertile genotypes (Levin 1983; Mikkelsen et al. 1996; Soltis and Soltis 1999, 2000). By increasing genetic diversity, hybridization may thereby release these individuals from the genetic constraints that prevented their crop parent from adapting to natural habitats. Although adaptation does not ensure subsequent invasion, the hypothesis of hybridization as an “invasiveness catalyzer” is supported by numerous examples (Ellstrand and Schierenbeck 2000). Second, the expression of resistance genes can release transgenic crop-wild plant hybrids from their major enemies, thereby enhancing their fitness. Enemy release has recently been demonstrated as a major cause of invasion in the case of exotic plants (Keane and Crawley 2002; Mitchell and Power 2003). Consequently, although enhanced fitness does not necessarily lead to invasiveness, genetically modified hybrids seem more likely to invade natural habitats than conventional hybrids.

To accurately assess the risks of the interspecific spread of transgenes, it is necessary to understand how natural habitats may be conducive to the establishment of transgenic crop-wild plant hybrids (Hails 2002). Enemy pressure is obviously a factor that may enhance the fitness of enemy-resistant hybrids relative to the fitness of their wild relatives: under high enemy pressure, resistant hybrids are expected to have higher fitnesses than severely damaged wild plants. In contrast, in the absence of enemy pressure, hybrids might have lower fitnesses than wild plants because of the potential deleterious effects of hybridization and resistance expression, which can be manifested by low pollen fertility (Jorgensen and Andersen 1994; Hauser et al. 1998a; Pertl et al. 2002) and/or low seed production (Chèvre et al. 1997). Plant density has recently been identified as an ecological factor that could magnify the effects of enemy selection pressure on the relative fitness advantage of resistant plants over susceptible relatives (Weis and Hochberg 2000). Consider, for instance, what would happen if enemies attack a dense stand of plants. Here, resistant individuals would be less damaged and would have access to the resources that their susceptible-damaged neighbors would have otherwise exploited. As such, resistant individuals not only escape attack, but they also capitalize on their neighbor's

misfortune. Conversely, plant density may also amplify the fitness costs of resistance and hybridization in the absence of enemies.

We studied the impact of these two potentially determining ecological factors—i.e. enemy pressure and plant density—on the initial spread of the most widely commercialized enemy-resistance transgene, the insect resistance gene from *Bacillus thuringiensis* (*Bt*). We grew mixed stands of wild plants and first-generation transgenic hybrids under different conditions of herbivore pressure and density, with *Bt* oilseed rape as the crop and *Brassica rapa* as the wild relative. We took biomass and fitness measurements of wild plants and *Bt* hybrids from their germination to the germination of their offspring. A crucial aspect of our study is that we also measured the frequency of resistant seedlings in the offspring generation. We address two main questions: (1) what are the effects of density and herbivory on the relative advantage of *Bt* hybrids over their susceptible relatives? and (2) what are the effects of density and herbivory on the frequency of *Bt* seedlings in the offspring generation?

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## Materials and methods

### Biological system

Oilseed rape (*Brassica napus* L. ssp. *oleifera*, AACC,  $2n=38$ ) is an ideal crop for studying the risk of transgene spread in natural habitats. It hybridizes easily with numerous wild relatives (Ellstrand et al. 1999), including birdseed rape (e.g. Jorgensen et al. 1996; Hauser et al. 1998b; Halfhill et al. 2002; Pertl et al. 2002). Birdseed rape (*B. rapa*, AA,  $2n=20$ ) is a common weed in many areas where oilseed rape is grown. Genetically modified lines of oilseed rape, transformed with an insecticidal *Bt* transgene, were supplied by Dr. Neal Stewart of the University of Tennessee and crossed with plants descending from a naturalized population of *B. rapa* found along the Back Bay, Newport Beach, California. To simulate the early phase of a *Bt* transgene escape into a wild *Brassica* population, we employed the resulting  $F_1$  *Bt*-transgenic hybrids as transgene donors and *B. rapa* plants as wild-type recipients. The genetically modified line of oilseed rape was homozygous for the *Bt*-transgene, whereas the  $F_1$  hybrids were hemizygous for the *Bt* transgene. The expression of the *Bt* gene gave both the genetically modified line and the  $F_1$  hybrids a high resistance to several defoliating insects, including the lepidopteran *Trichoplusia ni*.

Besides the *Bt cryIAC* gene from *Bt*, the introduced genetic construct contained a green fluorescent protein (GFP) gene (*mGFP5er*) under the control of the cauliflower mosaic virus 35S promoter, a nopaline synthase terminator cassette and a kanamycin resistance gene (neomycin phosphotransferase II, *nptII*) (Harper et al. 1999; Halfhill et al. 2001). Seedlings possessing the *GFP* gene show green fluorescence under UV light that is easily distinguished from the reddish-purple fluorescence of wild *Brassica* plants. Fluorescence intensity of  $F_1$  hemizygous hybrids is approximately half that of the homozygous parental lines but is still detectable under visual essays.

### Experimental design

The experiment was conducted in a greenhouse. The experimental design was a full factorial with plant density *D* (five levels,  $D1$ – $D5$ ) and insect herbivory *H* (three levels,  $H0$ – $H2$ ) as factors. Each possible treatment combination was replicated six times, giving a

total of 90 'microcosms'. Each microcosm was formed of a large pot (40 cm in diameter; 40 cm deep) filled with a 75/25 mixture of potting soil and sand, in which an equal number of  $F_1$  *Bt*-transgenic hybrids and *B. rapa* plants were grown. On the day of sowing, the microcosms were fertilized with a liquid fertilizer (N:K:P; 10:10:10).

In each microcosm,  $F_1$  *Bt*-transgenic hybrids and *B. rapa* plants were arranged on a checkerboard grid. Distances between adjacent plants equaled 12, 8, 6, 4 and 3 cm in the  $D_1$ ,  $D_2$ ,  $D_3$ ,  $D_4$  and  $D_5$  treatments, respectively, with the total number of plants on the grid being 4, 9, 16, 36 and 64 plants, respectively. These spatial configurations resulted in plant densities respectively equal to 55, 123, 219, 493 and 878 plants/m<sup>2</sup>. This range of densities was comparable to that observed in the natural populations from which the *B. rapa* plants originated (76–878 plants/m<sup>2</sup> with an average of 320 plants/m<sup>2</sup>) (D. Franke, personal communication). In order to minimize edge effects, we planted some of the plants between the edge of the grid and the pot borders. The stems of these plants were cut during the flowering period and did not contribute to reproduction nor data collection. In the lower density treatments ( $D_1$ – $D_3$ ), all the plants of the grid were tagged individually, whereas only two grid rows of plants were tagged in the highest density treatments (12 tagged plants in  $D_4$  and 16 tagged plants in  $D_5$ ). Thus, for each herbivory treatment and within each replicate, 57 plants were tagged. All of the tagged plants (a total of 1,026 plants) were checked weekly from May 2002 to September 2002.

In the  $H_0$  herbivory treatment, microcosms did not contain any herbivores. Low plant damage ( $H_1$ ) was obtained by carefully placing four first-instar caterpillars of *Trichoplusia ni* on each plant at the four-leaf stage. One week after this treatment, only 6% of the biomass, on average, had been removed from the most damaged leaf of the *B. rapa* plants. Plant damage by herbivorous insects might be that low in Back Bay (Newport Beach, Calif.) populations of *B. rapa* (A. Weis, personal observation). However, for our experiment to cover the full range of possible damage levels, high herbivory ( $H_2$ ) was simulated by removing the leaf blades of *B. rapa* plants at the four-leaf stage with a pair of scissors. In this treatment, the leaves of the hybrid plants were left intact.

#### Plant final biomass and statistical fit of the model

Five measurements of stem height (from the soil surface to the shoot apex) were made for each tagged plant from the second week after germination to plant death. Stem diameter (at the base) was measured on each tagged plant at the end of the growing season. Using a separately grown plant sample we verified that height  $\times$  width<sup>2</sup> is a good estimate of plant final biomass (Damgaard et al. 2002). Moreover, for each genotype and for each of the five density levels, we estimated the mean height of an individual in the  $H_0$  treatment. The height/biomass ratio was assumed to be constant over time. Growth increments were fitted to the model equation described in Weis and Hochberg (2000) using the NonLinearRegress routine of Mathematica (Wolfram 1999). The original equation was  $M_{t+1} = (M_t + \rho M_t) / [(1 + \theta_F M_t) (1 + \sum \delta_N^{-1} \theta_N M_{N,t})]$  where  $M_t$  is the biomass of the focal plant at time  $t$ ,  $\rho$  is the maximum growth rate,  $\theta_F$  is a constant depicting the effects of self-limitation (Isawa and Kubo 1997),  $M_{N,t}$  is the size of the neighbor  $N$ ,  $\theta_N$  represents the reduction in focal plant growth per unit biomass of the neighbor and  $\delta_N$  is the distance to the neighbor  $N$  (Weis and Hochberg 2000).

#### Flowering schedules and potential for interspecific matings

Twice a week we used a feather to transfer pollen en masse among plants within each microcosm. For the whole flowering period, the number of open flowers on each tagged plant was counted once a week. For each microcosm and each genotype, two variables were used to describe flowering schedules: the variable  $T_{iw}$  denotes the proportion of all flowers produced by genotype  $i$  over the season that were observed open in week  $w$ ; the variable  $G_{iw}$  is the

proportion of flowers produced by genotype  $i$  over all the flowers in the population that opened in week  $w$ . Under the assumptions that (1) each flower open in week  $w$  has the same probability of receiving and donating pollen and (2) each pollen grain has the same fertilization success, the mean probability  $\phi_{ij}$  that an ovule of a genotype  $j$  flower was fertilized by a pollen grain of a genotype  $i$  flower equals  $\sum_w G_{iw} T_{jw}$ . In addition, under the assumptions that (3)  $F_1$  hybrids produce an equal number of *Bt*-transgenic and susceptible pollen grains and (4) all the seeds have the same germination probability, the frequency of *Bt* offspring from wild-type mothers equals  $1/2 \phi_{TW}$  and the frequency of *Bt* offspring from *Bt*-transgenic mothers equals  $(1/2 + 1/4 \phi_{TT})$ , where  $T$  and  $W$  denote, respectively, transgenic and wild-type genotypes. The coefficient of assortative mating—i.e. the proportionate increase in within-type matings due to phenological differences between genotypes (see Li 1975)—was also calculated from flowering schedules for each microcosm (see Fox 2003).

#### Seed production

At the end of the growing season, pods were counted and collected for each tagged individual. Aggregate seed mass was measured for each of a subsample of 204 plants. Linear regression was used to estimate aggregate seed mass from pod number.

#### Germination rate and frequency of resistant seedlings

For each microcosm and each genotype, 32 seeds from tagged plants were arbitrarily chosen and sown. The proportion of seeds failing to germinate was recorded. Plants were screened at the four-leaf stage with a high-intensity, long-wave ultraviolet lamp. At this stage, green fluorescence was best visualized in the leaves and, particularly, in the vascular tissue (see Halfhill et al. 2001). Fluorescing offspring of *B. rapa* were hemizygous for the *Bt-GFP* transgene, whereas fluorescing offspring of the hybrid mothers could be either heterozygous or homozygous for it.

#### Statistical analysis

Effects of plant density and herbivore pressure were tested from mean genotypic values at the microcosm level. For each genotype and each microcosm, we noted mean values of the six following plant traits: final biomass, flower number, seed mass, germination rate, expected frequency of *Bt* offspring from flowering schedules and observed frequency of *Bt* offspring. The relative advantage,  $I_x$ , of *Bt*-transgenic hybrids ( $T$ ) over wild-type plants ( $W$ ) for trait  $x$  was defined as  $(X_T - X_W) / (1/2 X_T + 1/2 X_W)$ , where  $X_G$  is the mean trait value for genotype  $G$  (Weis and Hochberg 2000). Three additional data—the coefficient of assortative mating, the expected frequency of *Bt* offspring in the population and the observed frequency of *Bt* offspring in the population—were also available at the microcosm level.

All statistical analyses were conducted using SAS (1999). A factorial type I analysis of variance (SAS, PROC GLM) was conducted to study the effects of density and herbivory on the relative biomass and fitness advantage of *Bt*-transgenic hybrids over wild-type plants, on the frequencies of *Bt* offspring and on the coefficient of assortative mating. To test if the relative fitness advantage of *Bt*-transgenic hybrids over wild-type plants was significantly different from zero, we used a Student's  $t$ -test (SAS, PROC MEANS). Normality of the data was checked with the Kolmogorov-Smirnov goodness-of-fit procedure (SAS, PROC UNIVARIATE). An arcsin transformation was used to improve the normality of the frequencies of *Bt* seedlings in the offspring generation. However, this transformation did not result in a normal distribution, neither for the frequency of *Bt* seedlings in the offspring of wild-type mothers, nor the frequency of *Bt* seedlings at the population level. The effects of density and

herbivory were therefore separately checked with a Wilcoxon paired-sample test (SAS, PROC NPAR1WAY). All of the correlations (SAS, PROC CORR) presented were performed after having checked the normality of the data with the Kolmogorov-Smirnov goodness-of-fit procedure (SAS, PROC UNIVARIATE).

## Results

Effects of density and herbivory on the relative advantage of *Bt*-transgenic hybrids over wild-type plants

### Relative biomass advantage

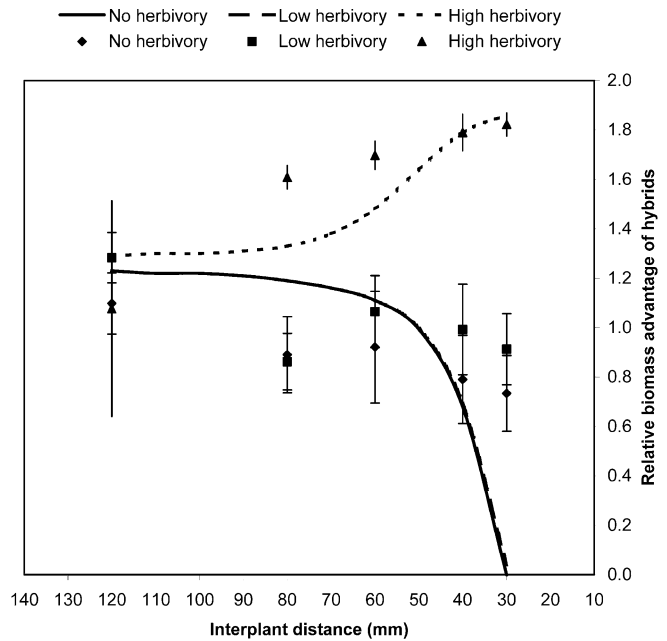
Because we found a significant effect of genotype ( $P=0.0093$ ) in the regression analysis (SAS, PROC REG) of final biomass (log-transformed) on height  $\times$  width<sup>2</sup> (log-transformed), we estimated this relationship separately for each genotype. Linear relationships were—for *B. rapa*,  $\log(\text{biomass})=0.30258+0.89716 \times \log(\text{height} \times \text{width}^2)$  ( $R^2=0.90$ ,  $P<0.0001$ ); for the  $F_1$  hybrid,  $\log(\text{biomass})=0.25899+0.92971 \times \log(\text{height} \times \text{width}^2)$  ( $R^2=0.98$ ,  $P<0.0001$ )—where biomass is in milligrams, height is in centimeters and width is in millimeters.

The interaction between herbivory and density had a significant effect on the relative biomass advantage ( $I_{\text{biomass}}$ ) of *Bt*-transgenic hybrids over wild-type plants ( $F=2.24$ ;  $P=0.03$ ; Table 1; Fig. 1). For all growing conditions tested, *Bt*-transgenic hybrids had a higher above-ground biomass than their wild-type competitors ( $I_{\text{biomass}}>0$ ; Fig. 1). The magnitude of this biomass advantage was sensitive to plant density and, as predicted by the Weis and Hochberg model, density amplified the biomass differences between resistant plants and their susceptible competitors (Fig. 1). The biomass advantage of *Bt*-transgenic hybrids was highest under high herbivory ( $H_2$ ) and high plant density ( $D_5$ ): the average biomass of hybrids ( $2,501 \pm 459$  mg) was then 19.8-fold higher than that of their wild neighbors ( $126 \pm 43$  mg).

The model equation developed by Weis and Hochberg (2000) was found to be a good descriptor of plant growth in a competitive environment (*B. rapa*:  $R^2=0.85$ ,  $P<0.0001$ ;  $F_1$  hybrid:  $R^2=0.80$ ,  $P<0.0001$ ). The best fits of the basic growth rate  $\rho$ , self-limitation constant  $\theta_F$  and competition coefficient  $\theta_N$  were—for *B. rapa*,  $\rho=2.2910$ ,  $\theta_F=0.0004$ ,  $\theta_N=20914.3$ ; for the  $F_1$  hybrid,  $\rho=2.3165$ ,  $\theta_F=0.0001$ ,  $\theta_N=23970.6$ —with biomass  $M$  in milligrams

**Table 1** Analysis of variance (ANOVA) (type I) of the relative biomass advantage of *Bacillus thuringiensis* (*Bt*)-transgenic hybrids over wild-type plants

Source	df	Mean square	F value	P
D, density	4	0.030	0.19	0.9422
H, herbivory	2	4.473	28.52	<0.0001
D $\times$ H	8	0.352	2.24	0.0337
Error	72	0.157		



**Fig. 1** Relative biomass advantage of *Bacillus thuringiensis* (*Bt*)-transgenic hybrids over wild-type plants as a function of herbivore pressure and interplant distance. Points represent experimental results and lines are model predictions

and interplant distance  $\delta_N$  in millimeters. Fitting was improved by assuming a non-linear effect of interplant distance,  $\delta_N$ , on plant growth ( $\delta_N^{-1}$  was replaced by  $\delta_N^{-5}$  in the model equation; see Discussion).

Therefore, additional simulations were conducted with these parameters and this modified model structure. Because 6% of the biomass on average had been removed from the most damaged leaf of *B. rapa* one week after the  $H_1$  herbivory treatment, biomass percentage removed from susceptible plants during herbivore attack was assumed to be 6% in the simulation of the  $H_1$  treatment. It was assumed to be 0 and 98%, respectively, in simulations of the  $H_0$  and  $H_2$  treatments. Herbivore-resistance of  $F_1$  hybrids was assumed to be 100%. Starting mass  $M_0$  was set at 100 mg. Linear regression (SAS, PROC REG) between experimental means for each treatment combination (log-transformed) and model predictions showed that the model explained 60% of the observed variance in the relative biomass advantage of hybrids ( $R^2=60.3$ ,  $P=0.0007$ ; Fig. 1).

### Relative fitness advantage

In the regression analysis (SAS, PROC REG) between seed mass (log-transformed) and pod number (log-transformed), we found a statistically significant effect of genotype ( $P<0.0001$ ) and herbivory ( $P=0.0156$ ), but no effect of density. Linear relationships were: for *B. rapa*— $\log(\text{seed mass})=1.79125+1.20853 \times \log(\text{no. of pods}) - 0.2464 H$  ( $R^2=0.81$ ,  $P<0.0001$ ); for the  $F_1$  hybrid— $\log(\text{seed mass})=0.63671+1.05941 \times \log(\text{no. of pods})$  ( $R^2=0.79$ ,  $P<0.0001$ ) where seed mass is in milligrams.

For the wild-type, plant final biomass was positively correlated to the three fitness components studied—flower number, seed mass and offspring germination rate (Table 2). Similarly, for *Bt*-transgenic hybrids, plant final biomass was positively correlated with flower number and aggregate seed mass, but not with offspring germination rate (Table 2). Despite the strong correlations between plant biomass and plant fitness, we did not find any significant effect of the interaction between herbivory and density on the relative fitness advantage of *Bt*-transgenic hybrids over wild-type plants for any of the fitness components.

The fitness advantage of hybrids in terms of flower production was not sensitive to density ( $F=0.59$ ,  $P=0.66$ ), but it was highly sensitive to high herbivory ( $F=70.88$ ;  $P<0.0001$ ; Fig. 2a). Similarly, the fitness advantage of the hybrids in terms of seed mass was not sensitive to density ( $F=1.67$ ,  $P=0.16$ ) but it did show a significant increase under high herbivory ( $F=111.40$ ;  $P<0.0001$ ; Fig. 2b). In contrast, the fitness advantage of the hybrids in terms of germination rate was not significantly sensitive to herbivory ( $F=1.31$ ;  $P=0.27$ ; Fig. 2c), but it did show a slight increase at high density ( $F=2.61$ ;  $P=0.04$ ). In the absence of herbivore pressure ( $H0$ ), *Bt*-transgenic hybrids had a significantly lower female fitness than their wild relatives (lower seed production and lower germination rate; Fig. 2), and there is a suggestion of a higher male fitness (higher flower production; Fig. 2). Under high herbivore pressure ( $H2$ ), *Bt*-transgenic hybrids had both a higher female fitness (higher seed production and a similar germination rate; Fig. 2) and a tendency towards a much higher male fitness (higher flower production; Fig. 2) than their wild relatives.

Effects of density and herbivory on the frequency of *Bt* seedlings in the offspring generation

#### *Frequency of Bt seedlings in the offspring of Bt-transgenic hybrids*

There was no significant effect of the interaction between herbivory and density on the frequency of *Bt* seedlings in the offspring of  $F_1$  hybrids. The mean frequency of *Bt* seedlings in offspring of hybrid mothers was 53%, and this value did not vary with density ( $F=0.49$ ,  $P=0.74$ ) nor herbivory ( $F=0.47$ ,  $P=0.42$ ).

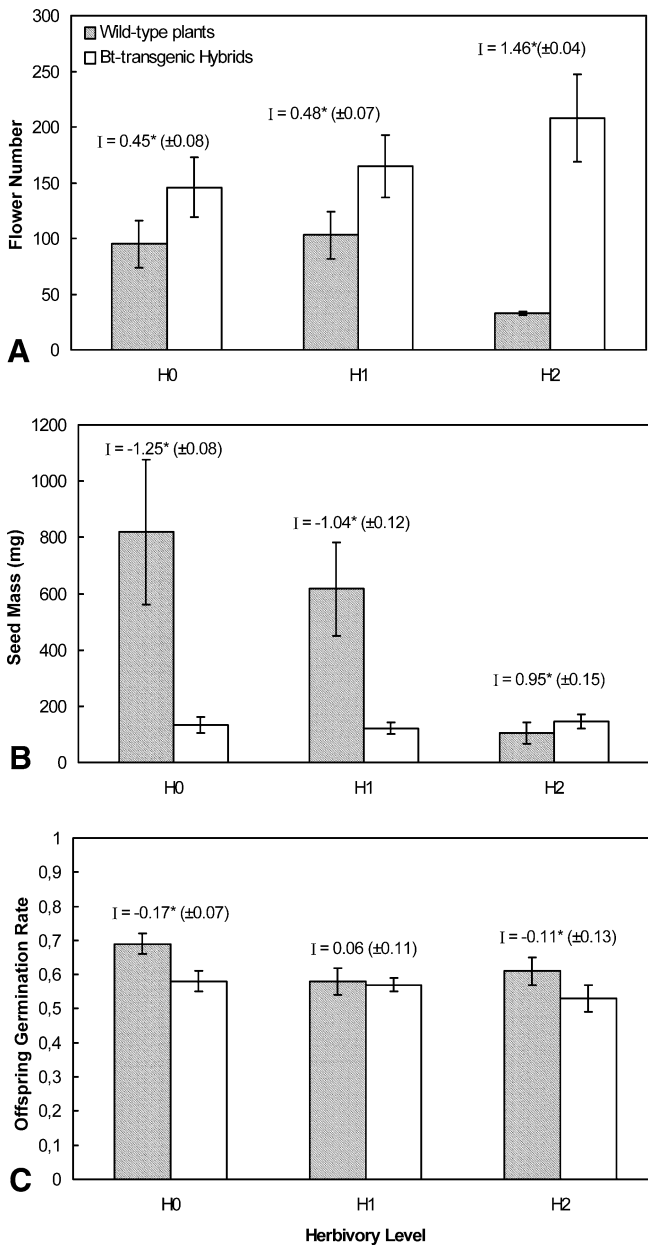
#### *Frequency of Bt seedlings in the offspring of wild-type plants*

Similarly, there was no significant effect of the interaction between herbivory and density on the frequency of *Bt* seedlings in the offspring of wild-type plants. However, there was a significant effect of density on the frequency of *Bt* offspring from wild-type mothers both by ANOVA ( $F=6.57$ ,  $P=0.0001$ ) and the non-parametric Wilcoxon paired-sample test ( $\chi^2=21.5$ ,  $P=0.0002$ ). The frequency of *Bt* seedlings in wild-type offspring was greater at high plant densities (Fig. 3b). The frequencies of *Bt* seedlings in the offspring generation predicted from flowering schedules were not correlated with the observed frequencies of *Bt* seedlings ( $R=0.02$ ,  $P=0.85$  for wild-type mothers;  $R=0.01$ ,  $P=0.91$  for *Bt*-transgenic mothers). Herbivore pressure tended to increase the frequency of *Bt* seedlings in the offspring of wild-plants (Fig. 3a), but the effect was not statistically significant.

**Table 2** Correlation analysis<sup>a</sup> of final biomass (log-transformed), flower number (log-transformed), seed mass (log-transformed) and germination rate for wild-type plants (in bold) and *Bt*-transgenic hybrids (in normal type)

	Final biomass	Flower number	Seed mass	Germination rate
Final biomass	–	<b><math>R=0.909</math></b> $P<0.0001$ $n=88$	<b><math>R=0.913</math></b> $P<0.0001$ $n=87$	<b><math>R=0.219</math></b> $P=0.04$ $n=88$
Flower number	$R=0.863$ $P<0.0001$ $n=89$	–	<b><math>R=0.866</math></b> $P<0.0001$ $n=87$	<b><math>R=0.195</math></b> $P=0.07$ $n=88$
Seed mass	$R=0.770$ $P<0.0001$ $n=89$	$R=0.866$ $P<0.0001$ $n=89$	–	<b><math>R=0.334</math></b> $P<0.01$ $n=87$
Germination rate	$R=0.048$ $P=0.65$ $n=89$	$R=0.038$ $P=0.72$ $n=89$	$R=0.055$ $P=0.61$ $n=89$	–

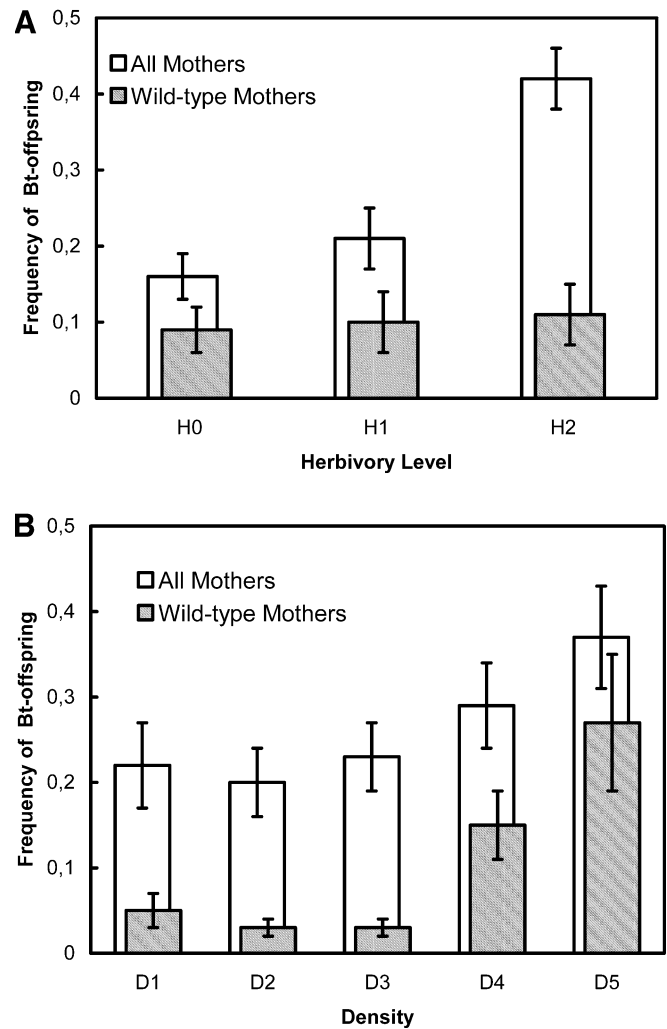
<sup>a</sup> $R$  is the Pearson correlation coefficient,  $P$  is the statistical significance of the correlation,  $n$  is the number of observations



**Fig. 2a–c** Fitness values ( $\pm$  standard error) of *Bt*-transgenic hybrids (white bars) and wild-type plants (grey bars) as a function of herbivore pressure for three fitness components. **a** Total number of flowers produced during the flowering period, **b** seed mass, **c** seed germination rate. *I* is the relative fitness advantage of *Bt*-hybrids over wild-type plants, asterisk indicates an advantage significantly different from zero

#### Frequency of *Bt* seedlings in the offspring generation at the population level

We did not detect any significant effects of the interaction between herbivory and density on the frequency of *Bt* seedlings in the offspring generation at the population level. A marginally significant effect of density on the frequency of *Bt* seedlings in the offspring generation was found by an ANOVA ( $F=2.44$ ;  $P=0.054$ ), but this not confirmed by the non-parametric Wilcoxon paired-sample



**Fig. 3** Frequency of *Bt* seedlings in the offspring of wild-type mothers (grey bars) and frequency of *Bt* seedlings at the population level (white bars) as a function of herbivore pressure (**a**) and plant density (**b**)

test ( $\chi^2=6.26$ ,  $P=0.18$ ). Consequently, the increase with density in the frequency of *Bt* seedlings in the offspring of wild-type plants translated into a weaker tendency for the frequency of *Bt* seedlings to increase at the population level (Fig. 3b). Finally, a significant effect of herbivory on the frequency of *Bt* seedlings in the offspring generation at the population level was demonstrated both by an ANOVA ( $F=17.68$ ,  $P<0.0001$ ; Table 3) and the non-parametric Wilcoxon paired-sample test ( $\chi^2=28.9$ ,  $P<0.0001$ ). Herbivore pressure strongly increased the frequency of *Bt* seedlings at the population level (Fig. 3a). This increase, and the tendency to increase observed in the frequency of *Bt* seedlings in the offspring of wild-plants (Fig. 3a), could be partly explained by a convergence in the phenologies of parental strains with herbivore pressure. Indeed, we found a significant decrease in the coefficient of assortative mating between *Bt*-transgenic hybrids and wild-type relatives with herbivore pressure ( $F=4.61$ ,  $P=0.01$ ).

**Table 3** ANOVA (type I) of the frequency (arcsin-transformed) of *Bt* seedlings in the offspring generation at the population level

Source	<i>df</i>	Mean square	<i>F</i> value	<i>P</i>
<i>D</i> , density	4	0.115	2.44	0.0544
<i>H</i> , herbivory	2	0.829	17.68	<0.0001
<i>D</i> × <i>H</i>	8	0.009	0.18	0.9923
Error	71	0.047		

## Discussion

The lines of *Bt*-transgenic oilseed rape and *B. rapa* employed here crossed readily and produced  $F_1$  hybrids with high fertilities and high backcrossing abilities (see also Halfhill et al. 2002). Our experiment shows that the fitness of genetically modified hybrids can even greatly overwhelm the fitnesses of their wild neighbors under certain ecological conditions. Under the conditions of high herbivore pressure and high plant density *Bt*-transgenic hybrids had a 20-fold higher biomass than their wild neighbors and produced 1.4-fold more seeds. Moreover, their seeds had similar germination rates to those of their wild neighbors. The frequency of *Bt* seedlings in wild-plant offspring reached 27% under high plant density, whereas 25% would have been expected under the assumptions of random mating and equal fertilities among plants. This means that the flower production advantage of *Bt*-transgenic hybrids compensated for their low pollen fertility (see Jorgensen and Andersen 1994; Hauser et al. 1998a; Pertl et al. 2002) and for decreases in intraspecific mating probabilities due to phenological differences between  $F_1$  hybrids and *B. rapa* plants.

Our conclusion—that in this *Bt* crop-wild plant system, first-generation hybrids do not constitute an important barrier to *Bt* transgene spread (see also Hauser et al. 1998b; Halfhill et al. 2002)—is based on two points. First, their high fertility suggests that stabilized hybrid lines may have a high potential for invasion. Second, our observations indicated that introgression is also a potential path for transgene spread. An important caveat, however, is that first-generation backcross and  $F_2$  hybrids might actually slow down the process of transgene spread, because of their low fitnesses, as shown by Hauser et al. (1998a). However, it should be noted that these researchers measured plant fitness in a non-competitive environment and without selection pressure for a transgene. Our experiment suggests that more complex environments could enhance the relative fitness of hybrid and backcross generations.

Indeed, the ecological factors studied in our experiment—herbivore pressure and plant density—had strong impacts on the relative biomass and fitness advantage of *Bt*-transgenic hybrids over wild-type plants. As previously predicted (Weis and Hochberg 2000), competition magnified the effect of herbivore pressure on the relative biomass advantage of resistant plants. The plant competition model developed by Weis and Hochberg (2000) with parameter estimates for the *Brassica* system examined

here was a relatively good predictor of final plant biomass under different conditions of density and herbivory. We improved its predictive accuracy by making two modifications to the initial model structure. First, since resistant and susceptible plants do not have the same genetic backgrounds in our system, growth and competition coefficients had to be estimated separately for the two parental strains. Second, the model assumed that the suppressive effect of neighboring plants is proportional to the inverse of inter-plant distance. Fitting our data to the model indicated that the competitive effect is proportional to a higher power of the inverse of inter-plant distance. This modification means that there is a distance threshold beyond which the suppressive effect of neighboring plants becomes negligible, which seems biologically reasonable. Such theoretical improvements based on experiments are crucial in developing models that can be used as tools of transgenic invasive hybrid management.

The same general patterns were observed for two major components of fitness—flower production and seed production—but for these traits the amplifying effect of competition was not statistically significant, despite the fact that biomass and fitness components were strongly correlated. Herbivore pressure remained the only significant factor when considering the relative fitness advantage of *Bt*-transgenic hybrids over wild-type plants. *Bt*-transgenic hybrids produced far more flowers than their wild neighbors, even in the absence of herbivore pressure. This relative advantage in flower production significantly increased under high herbivore pressure. Thus, the relative male fitness of hybrids—i.e. the proportion of offspring fathered by hybrids—might experience a strong increase under high herbivore pressure. As a consequence of their high flower production, *Bt*-transgenic hybrids also produced many more pods than their wild relatives. However, as their seed set per pod was very low (see also Hauser et al. 1998b), they produced 6.2-fold fewer seeds than wild-type plants in the absence of herbivore pressure. This cost of resistance and/or hybridization was fully compensated by the benefits of resistance under high herbivore pressure. As noted above, hybrids produced 1.4-fold more seeds than *B. rapa* plants in this treatment, and their seeds had similar germination rates. Therefore, the relative female fitness of hybrids—i.e. the offspring proportion mothered by hybrids—may also increase sharply under high herbivore pressure.

These dramatic effects of *Bt* transgenes on plant fecundity are also supported by the recent study of Snow et al. (2003) on  $BC_1$  wild sunflowers. Their study suggests that reduced herbivory by lepidopterans on transgenic plants could possibly cause *Bt* hybrids to produce twice as many seeds as conventional hybrids. Thus, the fitness effect have now been assessed for two major crop plants, and in both cases the inevitable escape of *Bt* transgenes from cultivated areas are predicted to have an impact on the ecology of their wild relatives. Our study goes further than that of Snow et al. (2003) in quantifying how the relative fitness of *Bt*-transgenic hybrids in the parental generation translated into *Bt*

seedling frequency in the offspring generation, by employing transgene monitoring with the *GFP* marker.

Unsurprisingly, we found that herbivore pressure strongly increased the frequency of *Bt* seedlings at the population level. *Bt*-transgenic plant frequency dropped from 50% to 16% within a single generation in the absence of herbivore pressure, but only to 42% under high herbivore pressure. This means that under high herbivore pressure, the frequency of the *Bt* transgene was greater or equal to 21% in the offspring generation, although it equaled 25% in the parental generation. Thus, herbivore pressure appeared as a selective agent strong enough to impede the decline of the *Bt* transgene driven by the cost of resistance and/or hybridization in terms of seed production experienced by *Bt*-transgenic hybrids.

Moreover, transgene monitoring with the *GFP* marker allowed us to gauge how ecological factors influenced the intensity of gene flow between the parental strains. Two points are notable. First, the introgression rate—i.e. the frequency of *Bt* seedlings within the offspring of wild-type plants—showed a significant increase at high plant density. Given this density effect on gene flow intensity, and given variations experienced by pollen and seed germination rates (Hauser et al. 1998a), the frequency of *Bt* seedlings among the offspring of each parental strain cannot be accurately predicted from Mendelian segregation fractions and mating probabilities based on flowering schedules. Second, the introgression rate showed a tendency to increase with herbivore pressure. This trend might be related to the increase in the relative flower number of *Bt*-transgenic hybrids under high herbivore pressure and to the increasing phenological convergence between *B. rapa* plants and *Bt*-transgenic hybrids with herbivore pressure. Additional studies are needed to understand how flower production variations and phenological shifts driven by ecological factors interact to determine introgression rates.

Finally, given the ample evidence for high hybridization efficiency between oilseed rape and *B. rapa* (see Jorgensen et al. 1996; Hauser et al. 1998b; Halfhill et al. 2002; Pertl et al. 2002), it is certain that  $F_1$  hybrids will emerge in natural habitats surrounding cultivated fields (Wilkinson et al. 2003). Our results suggest that the persistence of *Bt* transgenes in these habitats—through wild-plant introgression or hybrid stabilization—should be favored by ecological conditions such as high herbivore pressure and high plant density. However, using these greenhouse results to give a quantitative prediction of *Bt*-plants spread and persistence in natural habitats would not be sensible. First, our experiment is designed to identify the *local* conditions under which *Bt*-transgenes might spread in a patch of wild plants. Since plant density and herbivore pressure are variable in space and time, mean ecological conditions in *B. rapa* natural habitats are not very informative about the risk of a local invasion of *Bt* transgenes. Second, our experiment is aimed at predicting the ecological conditions favoring the *initial invasion* of *Bt*-transgenes in wild habitats. Ecological conditions favoring the persistence of *Bt*-transgenes in patches of

wild plants are not studied here and might be different. Third, since greenhouse conditions are very different from natural conditions in many aspects (light, temperature, water, nutrients), one must be very cautious when extrapolating results from the greenhouse to the wild.

Thus, we prefer to conclude with a qualitative interpretation of our results: high density patches of highly damaged wild plants are the most vulnerable to *Bt* transgene invasion. They should be monitored for early detection of transgene spread, thereby permitting possible management responses. Simulation modeling that takes environmental heterogeneity into account will be an essential tool in predicting the rate and the spatial patterns of *Bt* transgene spread and the implementation of these management strategies.

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