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Growth, productivity, and competitiveness of introgressed weedy *Brassica rapa* hybrids selected for the presence of Bt *cry1Ac* and *gfp* transgenes

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Abstract

Concerns exist that transgenic crop × weed hybrid populations will be more vigorous and competitive with crops compared with the parental weed species. Hydroponic, glasshouse, and field experiments were performed to evaluate the effects of introgression of *Bacillus thuringiensis* (Bt) *cry1Ac* and green fluorescent protein (GFP) transgenes on hybrid productivity and competitiveness in four experimental *Brassica rapa* × transgenic *Brassica napus* hybrid generations (F_1, BC_1F_1, BC_2F_1 and BC_2F_2). The average vegetative growth and nitrogen (N) use efficiency of transgenic hybrid generations grown under high N hydroponic conditions were lower than that of the weed parent (*Brassica rapa*, AA, 2n = 20), but similar to the transgenic crop parent, oilseed rape (*Brassica napus*, AACC, 2n = 38). No generational differences were detected under low N conditions. In two noncompetitive glasshouse experiments, both transgenic and nontransgenic BC_2F_2 hybrids had on average less vegetative growth and seed production than *B. rapa*. In two high intraspecific competition field experiments with varied herbivore pressure, BC_2F_2 hybrids produced less vegetative dry weight than *B. rapa*. The competitive ability of transgenic and nontransgenic BC_2F_2 hybrids against a neighbouring crop species were quantified in competition experiments that assayed wheat (*Triticum aestivum*) yield reductions under agronomic field conditions. The hybrids were the least competitive with wheat compared with parental *Brassica* competitors, although differences between transgenic and nontransgenic hybrids varied with location. Hybridization, with or without transgene introgression, resulted in less productive and competitive populations.

Keywords: *Bacillus thuringiensis*, *Brassica napus*, *Brassica rapa*, hybridization, insect resistance, introgression

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Introduction

Transgenic crop varieties are being increasingly grown in commercial agriculture, and concerns exist over the potential for transgene escape to wild relatives via the production of transgenic hybrid populations (Stewart *et al*. 2000; Wilkinson *et al*. 2003a). One potential negative consequence associated with the inadvertent production of transgenic weeds is an increase in fitness and invasiveness of the weed species. It is conceivable that hybridization and introgression of transgenes may modify the physiological and ecological characteristics of the weed species, and increase its competitiveness with cultivated crops. It has been suggested that gene flow and changes in fitness could be a particular problem with the oilseed rape crop species, *Brassica napus*

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L. (AACC, 2n = 38) (Jørgensen & Andersen 1994; Warwick *et al*. 1999; Snow 2002).

A large percentage (16% in the USA, 70% in Canada) of *B. napus* varieties now grown commercially is transgenic (James 2003; Warwick *et al*. 2003). Sexually compatible weedy relatives occur commonly in and near fields in which the crop is grown. Of these, *Brassica rapa* L. (AA, 2n = 20) is the most widespread and cosmopolitan weed (Holm *et al*. 1997). Experimental results have shown that gene flow can occur between the two species (Jørgensen & Andersen 1994; Mikkelsen *et al*. 1996a). Hybridization between transgenic *B. napus* and *B. rapa* has also been described, and transgenes have been shown to be expressed in the hybrids (Mikkelsen *et al*. 1996b; Metz *et al*. 1997; Halfhill *et al*. 2001, 2002, 2003a, 2004; Warwick *et al*. 2003). In backcrosses with transgenic herbicide-resistant *B. napus*, gene flow to *B. rapa* had no negative reproductive costs resulting from introgression when plants were grown in environmental growth chambers; and it was suggested that such transgenes might persist in weedy *B. rapa* populations (Snow *et al*. 1999). The first transgenic weed × crop hybrids (*B. rapa* × *B. napus*) have been discovered in a commercial agricultural field in Canada, in which F_1 hybrids were found to be herbicide resistant (Warwick *et al*. 2003).

In previous experiments, F_1 hybrids produced from the hybridization of *B. rapa* and *B. napus* were triploid (AAC; 2n = 29) (Metz *et al*. 1997; Halfhill *et al*. 2002; Warwick *et al*. 2003). After one backcross generation, the ploidy of the BC_1F_1 generation (as assessed by nuclear DNA content) shifted towards that of *B. rapa* (Halfhill *et al*. 2002). It was however, not identical to *B. rapa*, confirming that a small portion of the C genome (estimated to be 1 or 2 chromosomes) was present in the first meiotic division producing the BC_1F_1 plants. In subsequent backcross generations (BC_2F_1 and BC_2F_2), the trend towards the loss of genetic material continued and ploidy level was indistinguishable from that of the diploid *B. rapa* parental species. The diploid composition was stable after the intermating of BC_2F_1 individuals (Halfhill *et al*. 2003a) demonstrating that a diploid population can be reached after hybridization and two generations of backcrossing.

The degree of *B. napus* genetic introgression associated with the transfer of green fluorescent protein (GFP) and *Bacillus thuringiensis* (Bt) transgenes into multiple backcross hybrid generations with *B. rapa* has been quantified (Halfhill *et al*. 2003a). The GFP phenotype enabled the tracking of the potentially fitness-enhancing insecticidal Bt transgene in hybrid plants by simple visual observation (Harper *et al*. 1999). Using dominant AFLP (amplified fragment length polymorphic) markers, F_1 hybrid generations were found to contain 95–97% of the *B. napus*-specific markers. Subsequently, each successive backcross generation demonstrated a reduction of markers resulting in a 15–29% presence in the BC_2F_2 population when the ploidy level has returned to the diploid *B. rapa* level (Halfhill *et al*. 2002, 2003a). This range of frequency is greater than the 12.5% expectation, indicating a preferential transmission of *B. napus* chromosomes or markers during transgene introgression.

Even though substantial interspecific hybridization and introgression can occur in agricultural settings between *B. rapa* and *B. napus*, transgenic hybrid productivity (i.e. biomass accumulation and fecundity) and competitiveness compared to parental types are still not well characterized. We have taken three different approaches to assess the effects of the acquisition of an agriculturally relevant Bt *cry1Ac* transgene whose expression confers insect resistance in weedy *Brassica* hybrids: (i) hydroponic studies in a growth chamber were used to assess vegetative growth and nutrient use efficiency of transgenic hybrids, (ii) glasshouse and field studies were performed to determine the productivity of transgenic vs. nontransgenic hybrids in the absence or presence of insect selection pressure, and (iii) competition experiments were performed in agricultural fields at two locations to assess transgenic vs. nontransgenic hybrid interference on a crop.

In these experiments, we examined growth potential and competitiveness of four experimental *Brassica rapa* × transgenic GFP/Bt *B. napus* hybrid generations (F_1, BC_1F_1, BC_2F_1 and BC_2F_2) (Fig. 1). Selection was based solely on the GFP phenotype, which marked the presence of the Bt gene, in each generation, in order to compare transgenic hybrids to the parental types. The transgene-based selection criteria employed here also mimic a field situation with a putative fitness-enhancing transgene of high positive selective value. The hybrid generations differ in genomic composition, ranging from triploid (AAC) in the F_1 generation to diploid (AA) in the BC_2F_2 generation, as a result of progressive backcrossing to *B. rapa* (Halfhill *et al*. 2002). Seedling vigor and early growth responses, examined in the hydroponics experiments, may be important vegetative indices to describe how hybrid weed populations compete in the critical period of crop establishment or in early stages of intraspecific competition among seedlings of the parental weed species (Gressel 2002). Weed species often grow more actively than crops in the presence of high nutrient levels (Appleby *et al*. 1976; Liebl & Worsham 1987), and physiological differences in nutrient use among populations were quantified by determining growth response to high and low nitrogen (N) levels in a hydroponic system. Glasshouse and field experiments were performed to determine the growth and reproductive capacity of hybrids in the presence and absence of herbivory. Finally, competition with a model crop species (wheat, *Triticum aestivum* L.) was examined under field conditions. Combined, these experiments provided empirical data to evaluate the growth and competitiveness of hybrid and backcrossed hybrid populations compared to the original parental species.

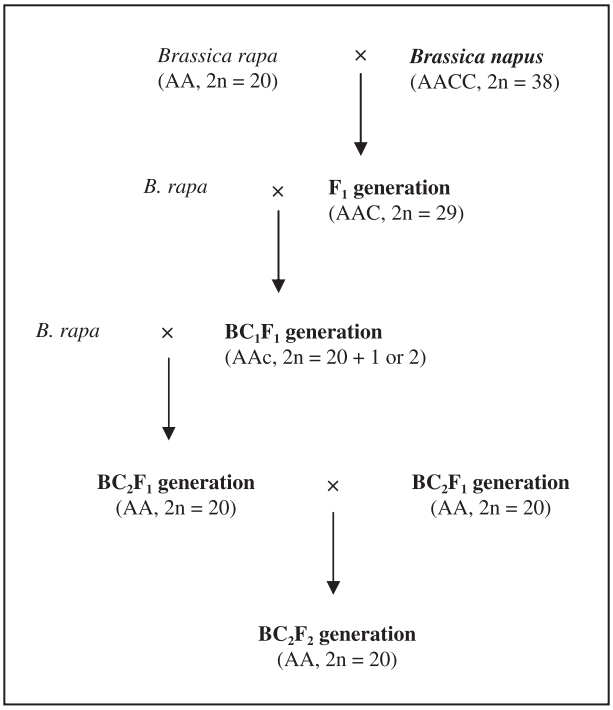

Fig. 1 The plant breeding pattern used to produce the hybrid generations. The maternal parent is indicated in the first position in each cross, and generations in bold were transgenic (GT = Bt/GFP). In the parentheses, letters represent genomic composition of the generation, and 2n number represents the chromosome number (Halfhill *et al*. 2002, 2003a).

Materials and methods

Plant breeding and description of plant genetics

Seven plant types (Halfhill *et al*. 2001, 2003a) were utilized in this study: *Brassica napus* (cv. 'Westar'), *B. napus* (cv. 'Westar') transgenic for green fluorescent protein (GFP)/*Bacillus thuringiensis* (Bt), *Brassica rapa* [wild-weed accession, 2974: Milby, Québec, Canada (45°19'N, 71°49'W), germplasm collection AAFC-ECORC, Ottawa], a self-incompatible species, and transgenic weed × crop hybrid generations (F_1, BC_1F_1, BC_2F_1, BC_2F_2) (Fig. 1). The transgenes were both under the control of the CaMV 35S promoter in separate cassettes on a single T-DNA vector: *mgfp5-er* and a synthetic truncated Bt *cry1Ac* (Halfhill *et al*. 2001). The pSAM12 plasmid (described in Harper *et al*. 1999) used to transform *B. napus* contained GFP-, Bt-, and kanamycin resistance cassettes in the T-DNA, enabling all three traits to be inserted in a single, genetically linked locus. Six plants of *B. rapa* were hand-crossed with pollen from one T_2 line (three plants) of transgenic *B. napus* (designated GT1, Halfhill *et al*. 2001) to generate a F_1 hybrid generation. Transgenic F_1 hybrid plants were selected in qualitative assays for GFP fluorescence using a hand-held, long-wave ultraviolet light (Spectroline high-intensity long-wave UV lamp, BIB-150P model, 350 nm), a reliable and robust method for selecting *gfp*-expressing transgenics (Stewart 2001). These transgenic hybrids were used as pollen donors and backcrossed with the parental *B. rapa* accession in the same fashion as above to produce a transgenic BC_1F_1 hybrid generation and subsequently a transgenic BC_2F_1 hybrid generation. The BC_2F_1 plants were intermated to produce a transgenic BC_2F_2 population by placing 27 individuals in a glasshouse enclosure with pollinating insects, houseflies (*Musca domestica* L.). Honeybees (*Apis mellifera* L.) could not be used in the glasshouse enclosures, and houseflies served as an effective substitute. A nontransgenic BC_2F_2 population was produced by crossing nontransgenic BC_2F_1 individuals in the same fashion. The GFP and Bt transgenes were shown to be genetically linked and functional through the BC_4 generation (Zhu *et al*. 2004a, b).

As indicated earlier, F_1 hybrids were triploid, while BC_1F_1 and BC_2F_1 had diploid ploidy levels and *B. napus*-specific AFLP markers (Halfhill *et al*. 2003a).

Hydroponic growth experiments

High N and low N growth experiments were conducted in four, 200-L continuous-flow hydroponic systems in the Southeastern Plant Environmental Laboratory at North Carolina State University (Downs & Thomas 1991). The environmental chamber was maintained at full intensity lighting with 8 h days at 18 °C/22 °C thermoperiods. Short days (8 h) were used to prevent premature flowering in the high intensity light of the environmental chamber. The total nutrient solution was based on a modified Hoagland's solution (200 μM KH_2PO_4, 300 μM $MgSO_4$, 800 μM $CaSO_4$, 35.8 μM Fe as Fe-EDTA, 19 μM H_3BO_3, 3.7 μM $MnCl_2{\cdot}H_2O$, 317 nM $ZnSO_4$, 132 nM $CuSO_4$, and 0.455 nM H_2MoO_4) with two N levels; high N (maintained at 1000 μM KNO_3) and low N (added daily to a level of 20 μM KNO_3). The nutrient solution was maintained at 20 °C and a pH of 6.0 ± 0.2 with automated monitoring and additions of KOH (0.01 M) and H_2SO_4 (0.01 M). For each trial, parental transgenic *B. napus* and *B. rapa* were grown as controls and compared to the growth response of two hybrid generations. The high N experiment was performed with transgenic *B. napus*, *B. rapa*, F_1, BC_1F_1, BC_2F_1, and BC_2F_2, and the low N experiment was performed with transgenic *B. napus*, *B. rapa*, F_1, and BC_1F_1.

The high and low N growth hydroponic experiments were conducted over 39-day periods. The high N experiment was conducted in two trials because of space limitations in the environmental chamber. In each trial, the parental species were grown with two transgenic hybrid generations (trial 1: F_1 and BC_1F_1; and trial 2: BC_2F_1 and BC_2F_2). Seeds were germinated in rolls of moist paper in 4-L beakers in 200 mL of 0.1 mM $CaSO_4$ solution to enhance root growth,

and were placed in a germination chamber at 26 °C for 72 h in the dark. Transgenic individuals were selected for the GFP phenotype on the germination paper by visual assay with a UV spotlight. Seedlings of uniform size (4–5 cm) were selected, and were transferred to the continuous-flow hydroponic system. Each hydroponic system initially contained 40 individuals of the same generation.

Plants were grown under 8-h day lengths, and remained vegetative and did not flower during the experiment (reproductive potential was not analysed). Five plants per generation were randomly harvested at 3-day intervals beginning on the 18th day post germination (eight harvests total). At each harvest, leaf area for each plant was recorded with a LI-COR 3100 leaf area meter (LI-COR Instruments). Total leaf area was calculated as the sum of all measurable leaves (those greater than 5.0 cm^2). Shoot and root tissues of each individual plant were separated into paper bags, and dried in a convection oven at 60 °C for 72 h. Dry weights per tissue type were recorded. The five plants in each harvest were bulked by shoot and root tissue. The bulked tissue was ground in a grinding mill, and analysed for N concentration with an automated CHN analyser (Perkin-Elmer 2400 or FlashEA 1112 Elemental Analyser, ThermoQuest Italia S.p.A.; software: EAGER 300 version 1.01). Total N accumulation per individual in each tissue type was determined by multiplying N concentration of the bulked tissue type by the dry weight of each individual (N concentration × tissue mass). Total N accumulation per individual was calculated by adding the N mass per tissue type (N mass shoot + N mass root). Nitrogen-use efficiency (NUE) was calculated as follows (total dry mass ÷ [N]), where [N] is nitrogen concentration in the shoot (Siddiqi & Glass 1981). At each harvest date, ANOVA was used to evaluate differences between plant types for each growth index. For the statistical analysis, plant type served as the treatment. Fisher's PLSD was used to determine if significant differences occurred between plant types (STATVIEW 5.0 for Windows 1992–1998; SAS Institute Inc.).

Glasshouse experiments

Productivity (vegetative dry weight and seed number per plant) of transgenic and nontransgenic hybrids was analysed in two glasshouse experiments at the University of Tennessee (Knoxville) in the spring of 2003 and 2004. A complete randomized block design was used, and plants were spaced to prevent competition. A colony of honeybees was maintained in the glasshouse to ensure pollination. Blocks contained five plant types (*B. rapa*, nontransgenic BC_2F_2, transgenic BC_2F_2, transgenic *B. napus* GT1 and nontransgenic *B. napus*). This design mimics field conditions where the different generations and crosses would be intermingled in a hybrid swarm. In year 1, four individuals per plant type (20 plants per block, 7 blocks, 140 total plants) were grown. In year 2, five individuals per plant type (25 plants per block, 6 blocks, 150 total plants) were grown. Plants were grown in 4-L pots under 20°/26 °C thermoperiods (10 h) and ambient photoperiods. Whole plants were harvested at maturity (completely yellow pods and before shattering), dried in the glasshouse, and above-ground dry weight recorded. Seeds were separated from the latter, and seed weight per plant determined. Seed number per plant was calculated by dividing total seed weight by 100 seed weight average. ANOVA was used to evaluate differences among plant types for vegetative dry weight and number of seeds per plant. Statistical analysis included treatment (plant type), block, and block × treatment interaction. Block × treatment interaction was not significant ($P > 0.20$), and was dropped from the analysis. Fisher's PLSD was used to determine if significant differences occurred among *Brassica* plant types (STATVIEW 5.0).

Field experiment — herbivory and intraspecific competition effects on productivity

Two field experiments were performed at the Knoxville Experiment Station, Plant Sciences Unit, Knoxville, Tennessee, USA (35°58′N, 83°55′W) in the spring of 2003 and 2004 under two herbivore levels and under high intraspecific competition levels. Five plant types (*B. rapa*, nontransgenic BC_2F_2, transgenic Bt BC_2F_2, transgenic *B. napus* GT1 and nontransgenic *B. napus*) were grown in 1-m^2 plots. Seeds of the appropriate type were scattered by hand in each plot at a density of 150 seeds per m^2. In 2003, nontransgenic BC_2F_2 seeds were not available, and were omitted from that year's field study. N-P-K fertilizer was added to recommended levels for agronomic *B. napus* production. After germination, the transgenic BC_2F_2 hybrid plots were screened with a hand-held UV light, and nontransgenic individuals were removed. The plant number in each plot was standardized to the plot containing the fewest individuals (year 1: 30 plants per m^2, year 2: 20 plants per m^2). Reduced herbivory: plots were sprayed with the insecticide endosulfan (Thiodan EC) at a concentration of 10 mL/L. Ambient herbivory: plots were not sprayed with insecticide. In year 1, *Brassica*-specific herbivores [Bt-susceptible diamondback moth (DBM), *Plutella xylostella* neonate larvae strain G88] were added to the ambient plots at an insect density of 165 neonates per m^2. No DBM survival or establishment was detected, and therefore insects were not added in year 2. In year 1, a total of 64 plots were sown (2 herbivore treatments × 8 replicate plots per treatment × 4 *Brassica* types) in a Latin square design. In year 2, a total of 100 plots were sown (2 herbivore treatments × 10 replicate plots per treatment × 5 *Brassica* types) in a Latin square design. During the growing season, herbivory damage on leaves was recorded using the following categorical damage scale (1 = no damage;

2 = < 1% damage; 3 = < 5% damage, 1 attempt; 4 = < 5% damage, > 1 attempt; 5 = 6–20% damage; 6 = 21–50% damage; 7 = 51–90% damage; 8 = > 90% damage). At maturity (completely yellow pods and before shattering), above-ground vegetative biomass was harvested in each plot separately, dried in the glasshouse, and dry weight per m² recorded. Statistical analyses were performed using a Latin square model with PROC MIXED (SAS version 9.1). Row location, column location, and plant type were listed as class variables. Plant type was classified as a fixed variable, with row and column classified as random variables. Least squares means were compared using pairwise *t*-tests.

Field experiment — interspecific competition with wheat

Interspecific competition between transgenic and nontransgenic hybrid generations and a model crop (wheat, *Triticum aestivum* cv. USG 3209, 82 cm variety) was analysed by establishing plots (1 m²) of four plant types (*B. rapa*, transgenic and nontransgenic BC_2F_2 hybrids, transgenic *B. napus*) under agronomic field conditions. The competition experiment was conducted at two separate sites: location 1, Central Crops Research Station, Clayton, North Carolina, USA (35°39′N, 78°27′W) from October 2001 through May 2002 and location 2, Lang Research Farm, Tifton, Georgia, USA (31°27′N, 83°30′W) from November 2002 through May 2003. N-P-K fertilizer was added to standard levels for agronomic wheat production. Wheat seeds were planted with a small row planter at row spacing of *c*. 15 cm and seeding rates of *c*. 75 seeds per metre. A complete randomized block design was utilized and five competition treatment types established: competition with *B. rapa*, transgenic or nontransgenic BC_2F_2 hybrids, transgenic *B. napus* GT1 and one control (wheat only — no *Brassica* competition). *Brassica* plants were either transplanted (location 1) or sown (location 2) in 1-m² plots for a final density of 15 *Brassica* plants per plot. A total of eight *Brassica* blocks (8 replicate blocks × 5 competition treatment types, 40 total plots in location 1) and 10 *Brassica* blocks (10 replicate blocks × 5 competition treatment types, 50 total plots in location 2) were randomly placed within the fields. In the spring of the following year at maturity (completely yellow stems), wheat in the 1-m² plots was cut at ground level, bagged, dried in the glasshouse, and then threshed to obtain seed mass. Wheat dry weight and seed mass were recorded per 1-m² plot and effects of competition quantified by comparing wheat yields and biomass among competition type. Statistical analysis included treatment (five competitive treatment types), block, and block × treatment interaction. Block × treatment interaction was not significant ($P > 0.05$), and was dropped from the analysis. Fisher's PLSD was used to determine if significant differences occurred among plant type (STATVIEW 5.0).

Results

High N hydroponic growth trials

All plants from each generation grew exponentially for the duration of the experiment under high N conditions. *Brassica rapa* accumulated more vegetative dry weight (i.e. shoot plus root weight) and had greater leaf area than the transgenic *Brassica napus* and the transgenic hybrid generations tested (Fig. 2) (Fisher's PLSD, $P < 0.05$). In trial 1, *B. rapa* had more vegetative dry weight than the hybrid generations (F_1 and BC_1F_1) and *B. napus* 30 days post germination (Fig. 2a) and more leaf area by day 27 (Fig. 2b). At the end of the trial, the average accumulated vegetative dry weight in the F_1 and BC_1F_1 hybrids was only 73% and 66% of that observed for *B. rapa*, respectively. There were greater differences in leaf area with the F_1 generation having on average 64% and the BC_1F_1 generation 57% as much leaf area as *B. rapa*. *B. napus* and the hybrid generations had similar growth rates in trial 1, with similar average leaf areas and vegetative dry weights at the end of the experiment.

The growth of all plants in trial 2 was slower in comparison to trial 1 (Fig. 2c, d). At the first harvest date (day 18) of trial 2, harvested plants were too small for accurate determination of leaf area, and were excluded from the study. *B. rapa* had significantly more vegetative dry weight than that of BC_2F_1 and BC_2F_2 hybrid generations starting on day 33 (Fig. 2c) and more leaf area on day 27 (Fig. 2d). The BC_2F_1 generation had the lowest average growth rates in comparison to the *B. rapa* parental line, with only 54% of the vegetative dry weight and 59% of the leaf area of *B. rapa* at the end of the trial. The BC_2F_2 generation plants also differed from *B. rapa* and accumulated 67% and 78% of the *B. rapa* vegetative dry weight and leaf area. In trial 2, *B. rapa* had more dry weight than *B. napus* on day 33 (later than trial 1), and day 27 (earlier than trial 1) for leaf area. In trial 2, *B. napus* had consistently more vegetative dry weight at each harvest date than the hybrid generations.

Total N accumulation and N-use efficiency were calculated for the high N growth experiment. *B. rapa* had a greater accumulation of N than hybrid plants (Fig. 3). *B. rapa* had more N than the F_1 and BC_1F_1 at day 30, and more N than the BC_2F_1 and BC_2F_2 generations at day 33. At the end of the study, the hybrid plants in all generations consistently accumulated less total N than *B. rapa*, with an average of 25% less in plants from the F_1 generation and 50% less in BC_2F_1 plants. These values are consistent with relative sizes of the plants. In trial 1, transgenic *B. napus* and the F_1 and BC_1F_1 generations had similar total N. In trial 2, *B. napus* accumulated significantly more total N than hybrid generations BC_2F_1 and BC_2F_2. Nitrogen-use efficiency was calculated based on the N concentration in the shoot. *B. rapa* plants were found to have higher N-use

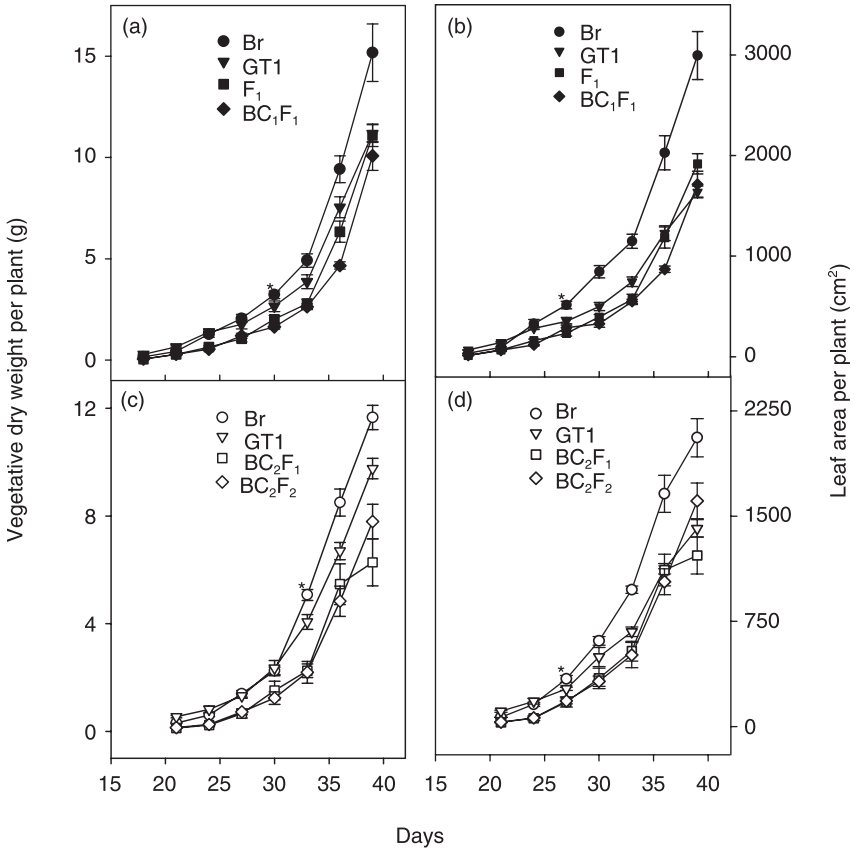

Fig. 2 Vegetative growth and leaf area of *Brassica rapa*, transgenic *Brassica napus* (GT1), and transgenic hybrid generations under high N conditions. Panels (a) and (b) represent trial 1, and panels (c) and (d) represent trial 2. The asterisk (*) represents the initial date when *Brassica rapa* (Br) significantly differed from *B. napus* GT1 and the hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2) (Fisher's PLSD, $P < 0.05$).

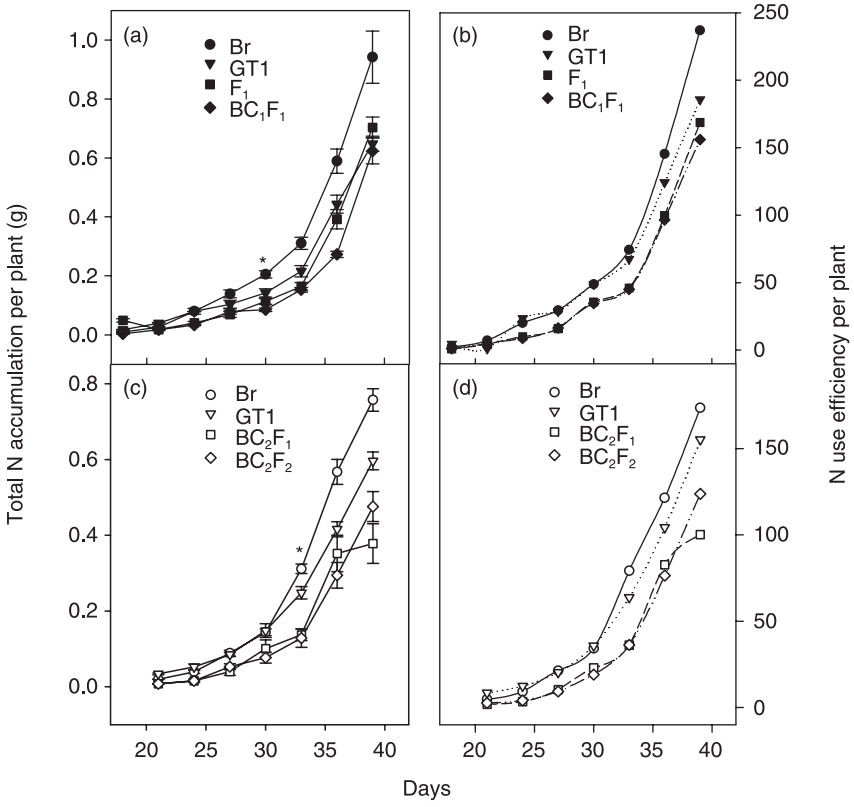

Fig. 3 Total N accumulation and N use efficiency of *Brassica rapa*, transgenic *Brassica napus* (GT1), and transgenic hybrid generations under high N conditions. Panels (a) and (b) represent trial 1, and panels (c) and (d) represent trial 2. The asterisk (*) represents the initial date when *B. rapa* (Br) significantly differed from *B. napus* (GT1) and the hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2) (Fisher's PLSD, $P < 0.05$). The parental species (Br and *B. napus* GT1) consistently demonstrated higher N use efficiency when compared to hybrid generations.

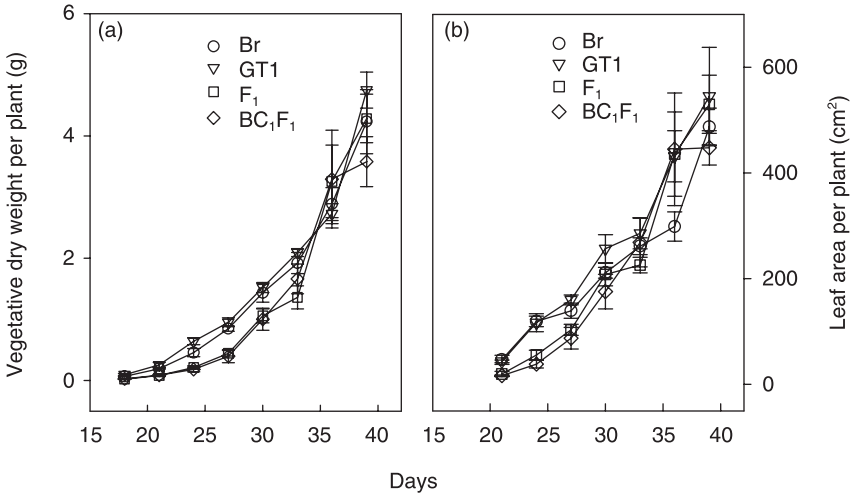

Fig. 4 Vegetative growth and leaf area of *Brassica rapa*, transgenic *Brassica napus* (GT1), and transgenic hybrid generations (F_1 and BC_1F_1) under low N conditions. Vegetative dry weight (a) and leaf area (b). Growth characteristics were generally similar between all generations.

efficiency than any of the four generations of hybrid plants (Fig. 3). In both trials, there was no difference between *B. rapa* and *B. napus* until day 30, and thereafter *B. rapa* had higher N-use efficiencies. The plants in the hybrid generations consistently had lower N-use efficiencies than the parental species in both trials.

Low N hydroponic growth trial

Differences in vegetative dry weight and leaf area among the transgenic F_1 and BC_1F_1 hybrid generations and the parental species (*B. rapa*, transgenic *B. napus*) were not significant at the end of the experiment (Fig. 4). *B. rapa* and *B. napus* grew at similar rates throughout the experiment, but differences were detected between the parental species and hybrid generations during the 24- to 30-day interval (Fig. 4). During this period, the parental lines accumulated on average significantly more dry weight than the hybrid generations (Fisher's PLSD, $P < 0.05$). These differences were overcome by day 33, and dry weight and leaf area were the same for all four plant types at day 40.

Productivity under glasshouse conditions

Plants of both transgenic and nontransgenic BC_2F_2 hybrid generations produced on average significantly less vegetative dry weight (Fig. 5a, c) and significantly fewer seeds per plant (Fig. 5b, d) than the *B. rapa* parent. No differences were evident between the transgenic and nontransgenic hybrid plants in year 1, but in year 2, transgenic BC_2F_2 hybrids had on average higher vegetative dry weight and seed production than the nontransgenic BC_2F_2 hybrids. Transgenic BC_2F_2 hybrid seed production was variable between years, with transgenic BC_2F_2 hybrids producing significantly more seeds per plant than *B. napus* in year 2. Plants of the two hybrid generations produced on average less vegetative biomass than *B. napus* in both years. *Brassica rapa* also produced on average significantly more seeds per plant than transgenic and nontransgenic *B. napus* (Fig. 5), indicating its greater reproductive potential.

Field experiment — herbivory and intraspecific competition effects on productivity

In year 1, lepidopteran larvae (susceptible to Bt) and other non-Bt susceptible herbivores [flea beetles, *Phyllotreta cruciferae* (Goeze) and *Phyllotreta striolata* F] were present in both treatments, and herbivore damage levels were not significantly different in the two herbivory treatments (i.e. with and without pesticide application). The insect resistance conferred by the Bt gene resulted in reduced herbivore damage in both the transgenic *B. napus* line and the transgenic Bt hybrid plants (Fig. 6). In year 2, lepidopteran larvae were not observed, and consistent early season damage on all plants (herbivory damage category 4) was primarily due to non-Bt susceptible herbivores (flea beetles). No differences were detected between the two herbivory treatments or among plant types (data not shown). In spite of the Bt herbivory protection advantage seen in year 1, Bt BC_2F_2 hybrids (both years), as well as nontransgenic hybrids (year 2), had on average less vegetative dry weight per m^2 than either parental species (Fig. 7).

Field experiment — interspecific competition with wheat

The competitive ability of transgenic and nontransgenic BC_2F_2 progeny and the two parental species was quantified by growing them in competition with cultivated wheat at two different sites in the southern USA. In each instance, *Brassica* plants were successfully established and grown at a density of 15 plants/m^2 within wheat fields under standard agronomic field conditions as winter/spring annuals. At

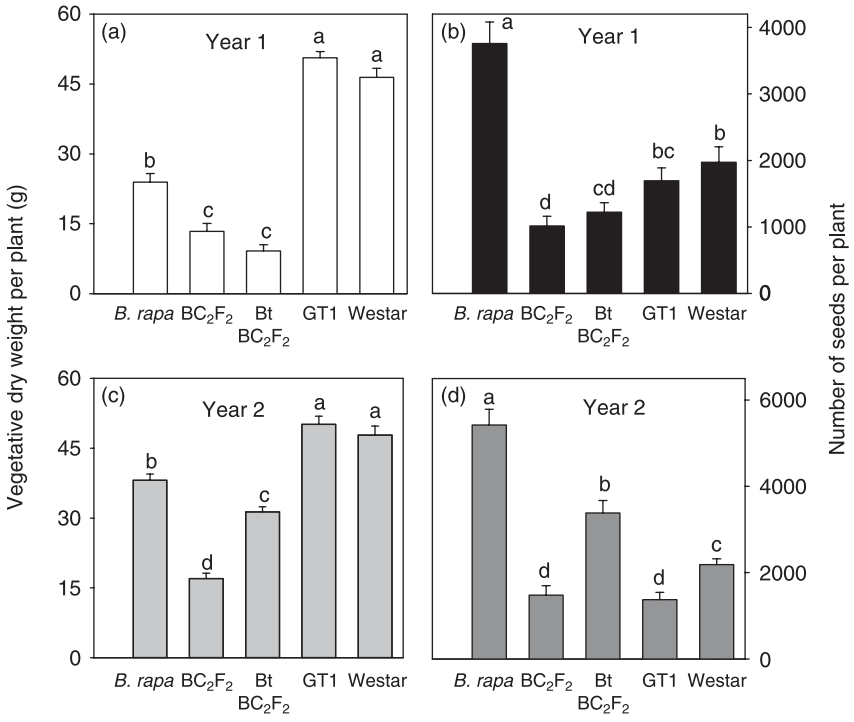

Fig. 5 Productivity of *Brassica rapa*, non-transgenic BC_2F_2 and transgenic Bt BC_2F_2 hybrid populations, and *Brassica napus* (transgenic GT1 and nontransgenic Westar) under glasshouse conditions. Panels (a) and (b) represent year 1, and panels (c) and (d) represent year 2. Different letters represent significant differences between treatments (Fisher's PLSD, $P < 0.05$).

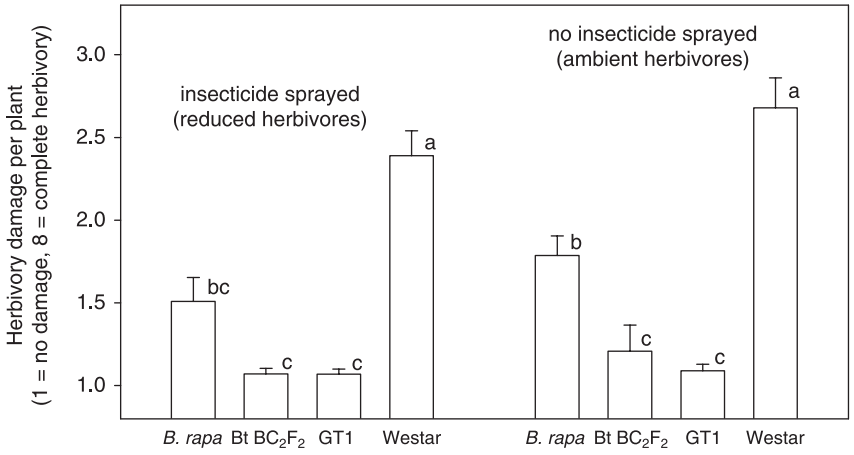

Fig. 6 Herbivory damage of *Brassica rapa*, transgenic Bt BC_2F_2 hybrids, and *Brassica napus* (transgenic GT1 and nontransgenic Westar) in year 1 under two field conditions: reduced herbivores (insecticide sprayed) and ambient insect levels (no insecticide sprayed). Herbivory damage as determined by visual assay. Different letters represent significant differences between treatments (Fisher's PLSD, $P < 0.05$).

both locations, the *Brassica* plants flowered in the early spring (March) and reached a height about 0.5 m taller than the stand of wheat. In all experimental plots at location 1, *Brassica* competition caused a reduction of wheat seed mass and vegetative dry weight (ANOVA, $P < 0.05$) (Fig. 8). At location 1 (NC), transgenic hybrids were the least competitive of the *Brassica* types tested (Fisher's PLSD, $P < 0.05$). Competitive growth of *B. napus*, *B. rapa*, non-transgenic BC_2F_2 and transgenic BC_2F_2 hybrid populations reduced wheat seed mass on average by 46%, 48%, 44% and 26%, respectively. Statistically similar results were found for wheat vegetative dry weight. At location 2 (GA), no significant differences in competitive ability were evident among plants of the four *Brassica* types, although no significant effect on wheat biomass was detected for non-transgenic BC_2F_2 hybrid plants compared to the control in either seed or dry weight (Fig. 8).

Discussion

Comparisons of hybrid types with parental species

For transgenes to persist in wild and weedy plant species, hybrids and backcrossed hybrids must be produced via intermating and then successfully compete with parental species and any other species that are in that community

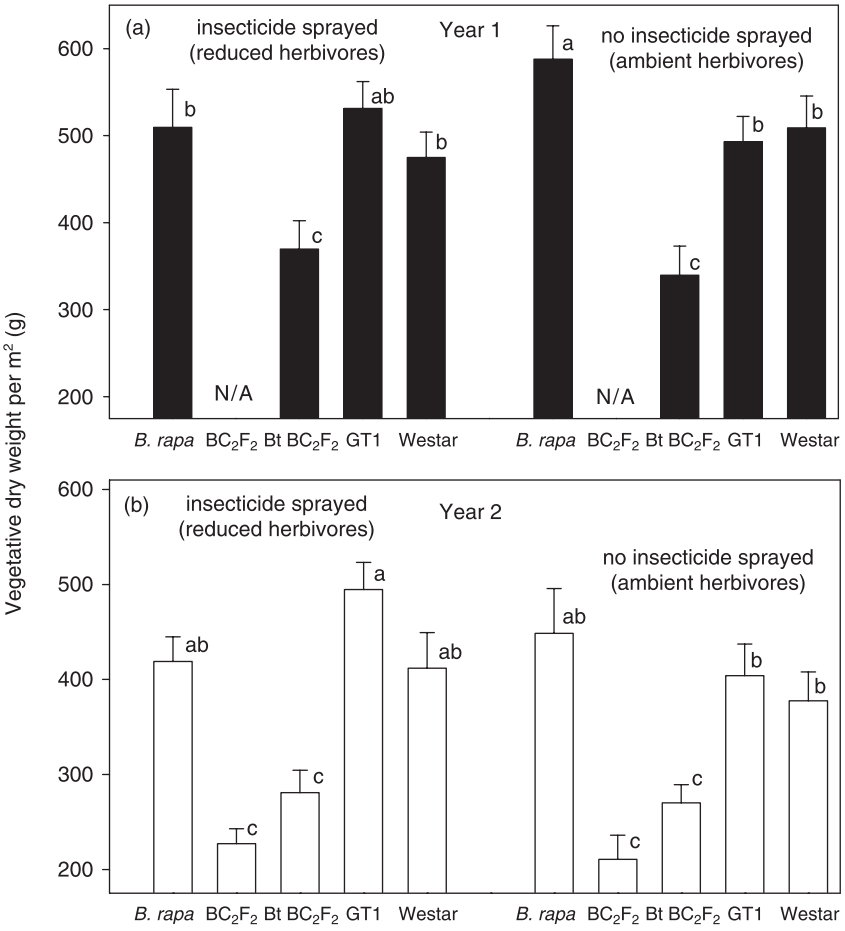

Fig. 7 Vegetative growth of *Brassica rapa*, transgenic Bt BC_2F_2 hybrids, and *Brassica napus* (transgenic GT1 and nontransgenic Westar) under two field conditions: reduced herbivory (insecticide sprayed) and ambient insect levels (no insecticide sprayed). Year 1 (a) and year 2 (b) show vegetative dry weight produced per m^2. Different letters represent significant differences between treatments (Fisher's PLSD, $P < 0.05$).

(Gressel 1999; Stewart *et al*. 2003). The results of the diverse experiments reported here demonstrate, that on average, transgenic and nontransgenic crop–weed hybrids of various backcrossed generations had lower potential for growth and competitiveness under field conditions than the weedy parental species. Weedy species generally respond in high nutrient environments with increased growth rates compared to competing crop species (Appleby *et al*. 1976; Liebl & Worsham 1987). The weedy nontransgenic *Brassica rapa* demonstrated the highest potential for vegetative growth rate when compared to plants of the transgenic hybrid generations, when grown under noncompetitive conditions. Hybrid generations did not exhibit increased growth rates over the parental weed species during the early vegetative stage when canopy development is likely to occur under high nitrogen levels. In comparison to the wild species, growth rates were consistently reduced from the triploid F_1 generation to the *B. rapa*-like diploid BC_2F_2 generation. Growth potential of the latter hybrids was not restored to the *B. rapa* level, even though they appeared to have the normal, diploid chromosome number (2n = 20) of the wild species. Hybrid growth, productivity, and competitiveness were more similar to *Brassica napus* than *B. rapa*. In summary, the backcrossed hybrid generations had the ploidy of the weedy *B. rapa* parent, but retained the physiological characteristics of the crop *B. napus* parent. This status may represent a diminished capacity for transgenic hybrids to compete with the same efficiency as the weedy parent.

Our selection for transgenic hybrid individuals did not intentionally generate populations with altered vegetative growth potential; vigor was neither selected for nor against — only the GFP transgene (hence Bt) was selected during the progressive formation of hybrid generations. Decreases in hybrid relative fitness and competitive ability could have been influenced by several factors, including linkage effects caused by the cointrogression of crop-adapted genes in hybrid generations, the expression and presence of transgenes, and other ecological factors.

Linkage effects

The physiological changes observed for the hybrid generations might be caused by the introgression of crop-adapted (*B. napus*) genes. Thus when a transgenic hybrid population is generated, genetic dilution of weed-adapted

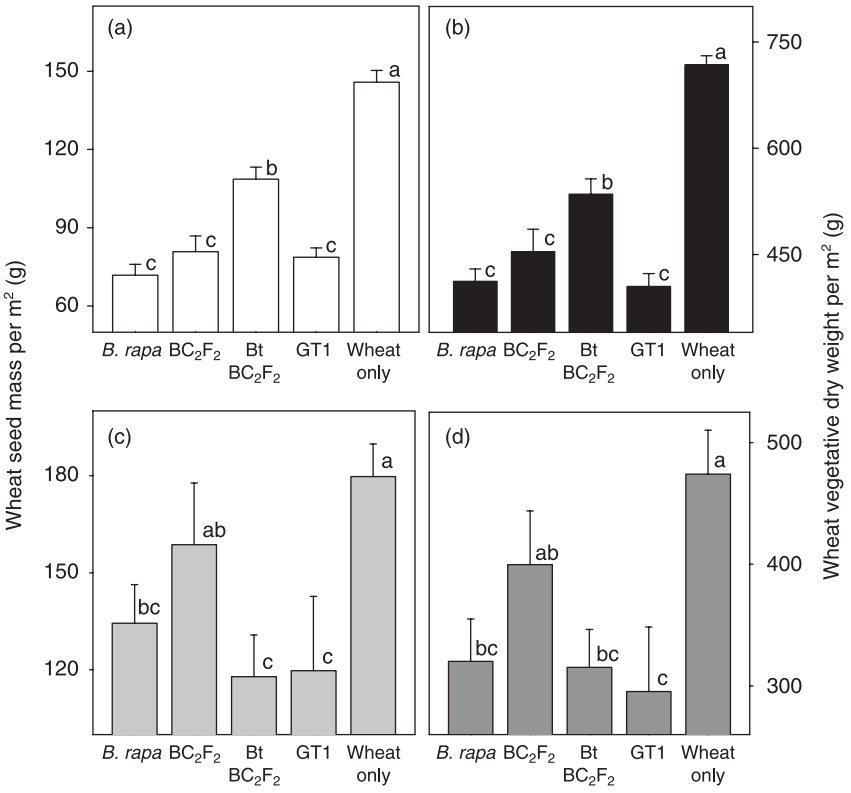

Fig. 8 Wheat seed mass and vegetative dry weight when grown in competition with various *Brassica* populations. Two locations of field data are presented: a and b, NC 2001–2002, c and d, GA 2002–2003. Four *Brassica* populations and one control were used: *B. rapa*; non-transgenic BC_2F_2 hybrids, transgenic Bt BC_2F_2 hybrids, transgenic *B. napus* (GT1), and wheat only with no *Brassica* competition. Different letters represent significant differences between treatments (Fisher's PLSD, $P < 0.05$).

genes could cause the average hybrid to pose less risk with regards to competitiveness as the wild parental species: a deviation of the assumption posed by many scientists and activists that transgenes in a weedy wild relative poses a special environmental risk (Stewart 2004). Indeed, backcrossing and transgene introgression will be affected by the transgene locus and by inherent properties of linkage disequilibrium (Stewart *et al*. 2003). It is apparent that during introgression, blocks of chromosomes are transmitted, and that this will vary between species pairs depending on the degree of chromosomal rearrangement (Burke *et al*. 1998; Rieseberg *et al*. 1999, 2000). There is experimental evidence in our transgenic system that this phenomenon is an important evolutionary force in introgression. Zhu *et al*. (2004b) found significant linkage distortion in certain Bt/GFP transgenic *B. napus* events during introgression. Three of nine such events showed lower than expected transgene transmission frequencies in controlled hand cross-pollinations (Zhu *et al*. 2004b). Even though the transgenic event GT1 used in this study was found to have Mendelian segregation during backcrossing, it is clear that locus and linkage effects are important factors that can be exploited to decrease transgene introgression from crop to weed (Stewart *et al*. 2003). In addition, environment and competition among hybrids and parental types are other factors creating barriers to introgression and transgene persistence (Pertl *et al*. 2002).

Transgene expression and locus

The presence and expression of transgenes could conceivably play a role in the reduced fitness of transgenic hybrid populations, especially experimental populations. Anecdotal reports that GFP may have cytotoxic effects in some transgenic events (Haseloff *et al*. 1997) are not supported by experimental data. When GFP tobacco was grown under field conditions, Harper *et al*. (1999) found that GFP production did not correlate to a reduction in plant biomass and seed production, demonstrating that at physiological concentrations GFP is a neutral marker under field conditions. The Bt Cry1Ac protein produced by the transgenic hybrid generations in this study is the same as produced in several transgenic varieties of field crops, such as cotton and maize. No specific reductions in yield have been demonstrated with the production of this protein. On the contrary, yield increases have been detected under agronomic conditions, presumably due to decreased herbivory (Graeber *et al*. 1999; Magg *et al*. 2001; Pray *et al*. 2002; Qaim & Zilberman 2003).

Transgenic-event-specific effects (position effects) can decrease fitness, and such events are usually discarded in crop breeding programs. Conceivably, if *gfp* and Bt *cry1Ac* transgenes were integrated into a locus of an endogenous gene, then physiological processes of the transgenic plant could be altered and fitness decreased. Position effects were not specifically tested in these experiments, and their

role in fitness may be elucidated in future research. The transgenic system used here has been characterized. GFP synthesis, the marker system for Bt expression levels, has been found to be additive in regards to transgene copy number in the transgenic event (GT1) used here and related transgenic events — GT2 to GT9 (Halfhill *et al*. 2003a). Zhu *et al*. (2004a) found that Bt synthesis was stable in F_1 to BC_4 *B. rapa* × *B. napus* hybrid generations and comparable to the levels in the original transgenic *B. napus* events (GT1 to GT9). There was no evidence for reduction of productivity in the various transgenic *B. napus* events, including GT1 of the present study, when tested under field conditions in the absence of herbivory (Mason *et al*. 2003).

Ecological factors

In order to predict the population dynamics of plants with and without the transgene, ecological interactions must be considered in measurements of potential fecundity, such as seed production. In these experiments, *Brassica* populations were selected on the basis of the probability that they will either be grown as a transgenic crop, or exist in an area where transgenic crops may be grown. We selected Canadian accessions in each case, and the discovery of naturally occurring hybrids containing a commercial herbicide resistance transgene in Québec, Canada, gives credibility to using this germplasm (Warwick *et al*. 2003). The field experiments reported here were conducted in the southern USA (Tennessee, North Carolina, and Georgia), where *B. rapa* occurs as a weed (USDA NRCS 2004) and *B. napus* is grown as a crop (Raymer *et al*. 2001). Growing Canadian varieties outside their normal geographical region may cause confounding patterns of growth. However, the *B. rapa* accession and *B. napus* variety used in this study have been grown in various locations in the southern USA with no apparent aberrations (Halfhill *et al*. 2002, 2003b, 2004). Although all plants, both hybrid and parental, were grown in the same conditions and compared to one another, the environmental conditions should be considered when analysing the conclusions of these experiments. The GFP phenotype may have also played a role in pollinator behaviour, as GFP fluorescence is detectable in flower tissues (Harper *et al*. 1999). Pollinator behaviour may be affected by GFP fluorescence in the transgenic flowers, and the GFP effect on pollination should be the focus of future research.

Future risk assessment studies should also include experimental selection for weedlike growth in transgenic hybrids in order to simulate natural selection under agricultural/ecological conditions. For the hybrid populations used in this study, the transgene was the only selection factor used to produce the populations, and the conclusions generated from this study must be viewed for the average member of the hybrid population. A selection regime for vigorous, weedlike individual hybrids may result in increased growth potential and competitiveness under certain ecological conditions. In similar studies, Hauser *et al*. (1998a) found that F_1 hybrids had intermediate fitness between *B. napus* and *B. rapa* based on several combined characteristics, and they concluded that F_1 hybrids were significantly more fit than *B. rapa*. In a subsequent study, Hauser *et al*. (1998b), found that a fitness depression occurred in F_2 and backcrossed individuals, although a small percentage of hybrids were as fit as the parents. The fitness of F_1 hybrids may also be frequency dependent (based on hybrid vs. parent ratio), and the experimental design in future research may need to include the appropriate ratio of hybrid to parental *B. rapa* plants to select for the hybrids with the highest fitness (Hauser *et al*. 2003).

Consequences of introgression

Previous studies have analysed multiple fitness components of transgenic hybrid generations from the crossing of herbicide resistant *B. napus* and *B. rapa*. Snow *et al*. (1999) found no significant differences in seed production between transgenic and nontransgenic BC_3 plants. They concluded that the cost associated with the herbicide resistance transgene (coding for glufosinate resistance) is negligible to hybrid populations based on reproductive indices. This laboratory-based study, however, did not consider early ecological stages, which is equally important for understanding population dynamics.

The lingering concerns about transgene movement from crops to weeds ultimately hinge on the ecological consequences of introgression and not introgression itself (Stewart *et al*. 2003; Poppy 2004). To date, only two studies have assessed reproductive fitness of crop × weed hybrids for putative fitness-enhancing transgenes — both with the common sunflower (*Helianthus annuus* L.). Snow *et al*. (2003) examined the fitness conferred by a Bt transgene in male-sterile BC_1 crop × wild sunflower hybrids in two separate field studies. They found apparent differences in crop × wild hybrid fecundity between sites; with $P = 0.054$ at one site, and at the other $P = 0.262$. Burke & Rieseberg (2003) examined the effect of a disease resistance transgene (coding for oxalate oxidase, *OxOx*) on the fitness of BC_3 wild sunflower hybrids. Even though this study did not examine competition effects, the authors used three field sites and advanced backcrossed material. Under *Sclerotinia sclerotiorum* (white mold) pathogen pressure, the transgene protected BC_3 plants from disease, but did not increase their reproductive fitness. Thus the authors predicted that such a fitness-enhancing transgene would 'diffuse neutrally' in time and space after escape from a crop genetic background (Burke & Rieseberg 2003).

Even though there are no compelling data to suggest that the use of transgenes is inherently risky, the findings of our study and the others discussed above might not

fully describe the risks posed by transgenic hybrid populations under field conditions, since they used single transgenic events and a limited number of hybrid families. We concur with Burke & Rieseberg (2003) that future studies should also examine the effects of genetic background and environmental stresses. Future experiments should be performed under field conditions that incorporate selection pressure and competition among hybrids with different genetic backgrounds and examine how additional or other crop markers sort during introgression. One benefit of such an approach will be to determine if transgene placement into specific loci might be exploited in transgenic crop development as a mitigation measure to decrease risk of introgression (Gressel 1999; Al-Ahmad *et al*. 2004).

If the number of GM crops continues to expand rapidly, risk assessment needs to become more focused (Wilkinson *et al*. 2003b). The protocols developed here could be used to identify and characterize the hazards (consequences) of gene flow into hybrids and wild relatives, and most importantly allow predictions to be made about populations based on genetic and ecological information. As risk assessment studies of GM crops become more advanced, they will need to become more predictive and holistic, and address the numerous factors influencing risk.

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