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Bt-transgenic oilseed rape hybridization with its weedy relative, *Brassica rapa*

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The movement of transgenes from crops to weeds and the resulting consequences are concerns of modern agriculture. The possible generation of "superweeds" from the escape of fitness-enhancing transgenes into wild populations is a risk that is often discussed, but rarely studied. Oilseed rape, *Brassica napus* (L.), is a crop with sexually compatible weedy relatives, such as birdseed rape (*Brassica rapa* (L.)). Hybridization of this crop with weedy relatives is an extant risk and an excellent interspecific gene flow model system. In laboratory crosses, T_3 lines of seven independent transformation events of *Bacillus thuringiensis* (*Bt*) oilseed rape were hybridized with two weedy accessions of *B. rapa*. Transgenic hybrids were generated from six of these oilseed rape lines, and the hybrids exhibited an intermediate morphology between the parental species. The *Bt* transgene was present in the hybrids, and the protein was synthesized at similar levels to the corresponding independent oilseed rape lines. Insect bioassays were performed and confirmed that the hybrid material was insecticidal. The hybrids were backcrossed with the weedy parent, and only half the oilseed rape lines were able to produce transgenic backcrosses. After two backcrosses, the ploidy level and morphology of the resultant plants were indistinguishable from *B. rapa*. Hybridization was monitored under field conditions (Tifton, GA, USA) with four independent lines of *Bt* oilseed rape with a crop to wild relative ratio of 1200:1. When *B. rapa* was used as the female parent, hybridization frequency varied among oilseed rape lines and ranged from 16.9% to 0.7%.

Key words: transgene, oilseed rape, *Brassica rapa*, hybridization, *Bacillus thuringiensis.*

INTRODUCTION

Transgenic crops are rapidly becoming a staple of modern agriculture. Since 1992, the USDA has deregulated fifty-two lines of transgenic crops for commercial field release (APHIS Permits 2000). The perceived ecological risks associated with the introduction of novel genes into ecosystems have led to controversy. Crop plants with weedy wild relatives are of particular concern. Hybridization between closely related species can be a mode of transgene flow directly into wild populations (Raybould and Gray, 1993). If expressed in the genetic background of a weed species, a transgene could conceivably change the fitness of the weed in nature. In the worst-case scenario, the weed could become more invasive and competitive.

The possibility for increased fitness of transgenic hybrids and backcrosses depends on the nature of the transgene. For example, weeds containing a transgene that confers resistance to an herbicide would be a nuisance to agriculture, but would have little effect in a non-agricultural environment where the herbicide is absent. In contrast, an insecticidal *Bacillus thuringiensis* (*Bt*) transgene in a weed host could alter natural ecology by giving transgenic weeds a selective advantage as the result of natural insect pressure (Stewart et al., 1997). This protein is toxic to many species of herbivorous insects, and is nontoxic to many other insects, birds, and mammals. The specificity of *Bt* endotoxins has made them attractive insect resistance transgenes that will be

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transformed into many agriculturally-important crop species. Transgenes that provide fitness enhancing characteristics under natural conditions have the potential to disrupt the balance of established ecosystems (Warwick et al., 1999).

Oilseed rape (*Brassica napus* (L.)) is an ideal crop for the study of transgene escape into wild relatives. Oilseed rape is an allotetraploid (AACC, 2n = 38) and has many wild weedy relatives such as birdseed rape (*Brassica rapa* (L.), AA, 2n = 20) and wild radish (*Raphanus raphanistrum*, RrRr, 2n = 18) persisting in or near areas of cultivation. Birdseed rape is a common weed in many places where oilseed rape is grown (Holm et al., 1997), and wild radish is a noxious cosmopolitan weed that can exist outside the agricultural setting. These weeds have been shown to hybridize with oilseed rape under both laboratory and field conditions (Chevre et al., 1997, 2000; Darmency et al., 1998; Eber et al., 1994; Jorgensen and Andersen, 1994; Lefol et al., 1996; Metz et al., 1997; Mikkelsen et al., 1996; Reiger et al., 1999). Also, oilseed rape transformation protocols have been developed and transgenic varieties have been widely studied under field conditions (Darmency et al., 1998; Paul et al., 1995; Ramachandran et al., 2000; Stewart et al., 1997). Oilseed rape has been transformed with fitness enhancing transgenes such as herbicide, disease, and insect resistance (Metz et al., 1997; Stewart et al., 1996b; Harper et al., 1999).

Transgenic hybrids have been produced between transgenic oilseed rape modified with herbicide resistance genes and *B. rapa* (Metz et al., 1997; Mikkelsen et al., 1996). After one backcross generation, many of the progeny were morphologically and cytologically similar to the *B. rapa* parent (Metz et al., 1997). After successive backcrosses into the weedy parent, it was found that 10% of the subsequent BC3 and BC4 hybrids had resistance to the herbicide (Metz et al., 1997). This illustrates that a transgene can be passed between species and be active in successive generations. To this time few studies have been performed to produce transgenic hybrids that would have a putative selective advantage outside agriculture.

Bt-transgenic oilseed rape has been generated and confers selective advantage in the presence of *Brassica* defoliating insects (Stewart et al., 1996b; Stewart et al., 1997). In an agricultural setting with moderate infestation of diamondback moth (*Plutella xylostella*), nontransgenic plants had lower fitness compared to transgenic oilseed rape producing the *Bt* endotoxin (Stewart et al., 1997). However, this selective advantage did not cause the transgenic oilseed rape to become more invasive outside the agricultural setting. In the genetic background of naturally occurring weeds, the *Bt* toxin could have a different effect. *Bt*-transgenic oilseed rape has the potential to pass this transgene and increase the fitness of naturally occurring weeds through hybridization.

The goal of this study was to monitor the production of *Bt* transgenic hybrids that could exhibit an insecticidal phenotype outside agriculture. The first objective was to compare the expression of the *Bt* transgene in the genetic background of the crop and weedy relative. Several T_3 lines from independent transformation events of *Bt* oilseed rape that differed in their transgene expression were used to monitor how recombinant protein production changes after hybridization occurs with a wild relative. The second objective was to compare hybridization and introgression rates among independent transgenic lines. The genomic location of transgene insertion has been proposed to play an important role in the ability of a genetically modified crop to pass transgenes to wild relatives (Metz et al., 1997), and hybridization experiments were performed with independent transformation events to detect differences in the transfer of transgenes to wild relatives. The third objective was monitor gene flow at the agricultural field level.

RESULTS

Growth chamber hybridization

Six of the seven *Bt* oilseed rape lines when crossed with *B. rapa* generated plants that survived antibiotic screening (Tab. 1). The California accession (CA) of *B. rapa* was able to produce seeds in all seven crosses, while the Montana accession (MT) did not produce seeds in crosses with O52, W63, and O124. One low *Bt*-expressing oilseed rape line, W58, produced no surviving plants when hybridization was attempted with the *B. rapa* accessions. When the seeds were germinated on media containing 50 $mg.L^{-1}$ hygromycin, the survivors grew similarly to the transgenic oilseed rape parents by developing long roots and producing leaves. The non-transgenic plants had little root elongation and turned purple as the result of stress caused by hygromycin toxicity. The surviving plants were moved to soil and characterized on the basis of morphology. The putative hybrids contained a range of characteristics that combined the traits of the two parents, such as number of trichomes and leaf shape (data not shown).

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Table 1. Production of hybrids between *Bt* oilseed rape and *B. rapa* under laboratory conditions.

	F_1 Hybrids[1] (transgenic/germinated)	BC_1 Hybrids (transgenic/germinated)	BC_2 Hybrids (transgenic/germinated)
B. rapa Montana × W45	33/35	14/32	8/40
B. rapa California × W45	1/7	4/124	4/124
B. rapa Montana × O48	9/15	7/63	11/74
B. rapa California × O48	4/13	4/29	18/80
B. rapa California × O52	4/20	0/99	-
B. rapa Montana × W58	0/4	-	-
B. rapa California × W58	0/24	-	-
B. rapa California × W63	6/21	0/149	-
B. rapa Montana × O96	0/6	-	-
B. rapa California × O96	5/9	0/107	-
B. rapa California × O124	2/4	4/59	19/80

[1] Seven T_3 lines of *Bt* oilseed rape were hybridized and backcrossed with two accessions of *B. rapa*. The oilseed rape was used as the pollen donor, and seeds were collected from the *B. rapa* parent. The seeds were germinated on sterile media containing 50 $mg.L^{-1}$ hygromycin to select for transgenic plants. For each of the crosses, three oilseed rape plants were crossed with six *B. rapa* plants. W and O represent oilseed rape cultivars Westar and Oscar, respectively.

Backcrosses

In the first backcross between *Bt*-transgenic hybrids and *B. rapa*, only three of the six F_1 hybrid lines were able to produce transgenic plants in the BC_1 generation (Tab. 1). W45, O48, and O124 produced transgenic plants at similar rates compared to the original hybridization event, while lines O52, W63, and O96 produced no plants that survived hygromycin selection. The morphology of the BC_1 plants was more similar to the characteristics of *B. rapa*. The leaves were more triangular in shape and had many trichomes, similar to *B. rapa*. All surviving transgenic BC_1 lines were able to produce transgenic BC_2 plants in the second backcross (Tab. 1). The morphology of all the BC_2 plants was indistinguishable from the *B. rapa* parent plants.

PCR analysis

DNA was isolated from all plants that survived antibiotic selection, and PCR was performed with *Bt*-specific primers. All surviving plants of the F_1 generation displayed a 550 base pair band and illustrated that the *Bt* gene was passed from the *Bt*-transgenic oilseed rape lines (Fig. 1). No positive PCR product was generated from control *B. rapa* plants. In the backcrosses, all surviving BC_1 and BC_2 plants also yielded the positive PCR product.

Protein analysis

Protein was extracted from the surviving plants and characterized by Western blot analysis. Hybrid plants (F_1) exhibited similar levels of expression compared to the parental oilseed rape line (Fig. 1). Moderate to high-expressing lines, such as W45, O48, and O124, yielded hybrids that produced *Bt* toxin protein at approximately 0.1% total soluble protein. The low expressing line, W63, produced low-expressing hybrids that had *Bt* toxin protein levels below the level of detection of the assay (less than 0.1 ng *Bt* toxin/µg protein extract). After back-crossing the toxin-producing hybrids with *B. rapa*, the BC_1 and BC_2 plants also expressed the *Bt* gene at similar levels as the original oilseed rape line. There was no

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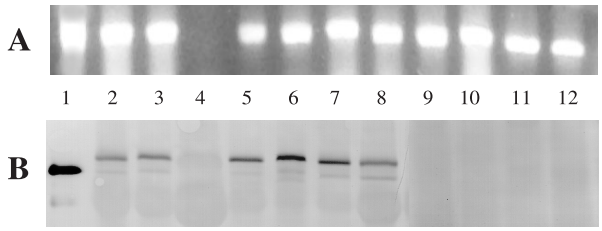

Figure 1. Panel A (PCR analysis) and Panel B (*Bt* CrylA*c* protein blot analysis) of transgenic hybrids of *Brassica rapa* and *Bt*-transgenic oilseed rape. Lane 1 contained *Bt* plasmid (Panel A) and 20 ng purified *Bt* toxin protein (Panel B). Lanes 2 and 3 contained the parental transgenic oilseed rape lines, W45 and O48 respectively. Lane 4 contained the wild relative *B. rapa* parent. Lanes 5–8 contained the resultant hybrids of the cross between a medium-high expressing transgenic oilseed rape line (W45), and lanes 9–12 are resultant hybrids of the low expressing event (W63). The minimum level of detection in the protein blot was 2 ng *Bt* toxin protein.

detectable difference in the level of expression of the transgenic hybrids and backcrosses compared to the original parental transgenic oilseed rape lines.

Ploidy determination

Flow cytometry analysis of the F_1 hybrids mixed with both parental species, respectively, illustrated that the F_1 hybrids had an intermediate DNA content between both parents (Fig. 2). The G1 peak (2C) of the F_1 hybrids demonstrated a lower DNA content than the oilseed rape peak (AACC, 2n = 38), but was above the *B. rapa* peak (AA, 2n = 20). The ploidy level of the F_1 hybrids is believed to be triploid (AAC), with a 2n number of 29 chromosomes, which is consistent with the flow cytometric data.

The peaks of BC_1 generation shifted toward the lower DNA content of *B. rapa*. However, these peaks were not identical with *B. rapa* (Fig. 2). This

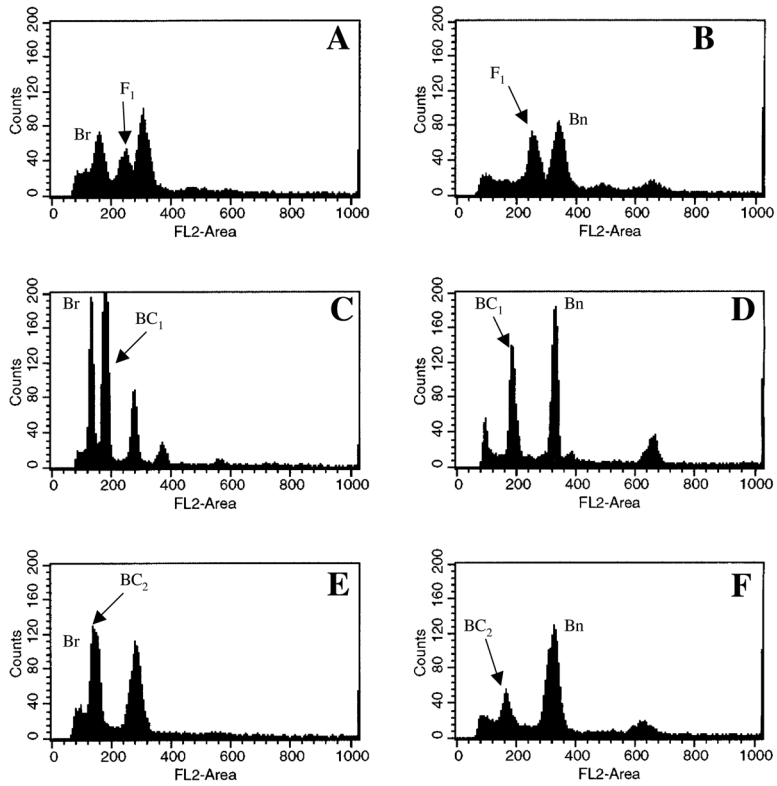

Figure 2. Relative DNA content of F_1, BC_1, and BC_2 hybrids. Histograms of flow cytometric analyses of nuclei of mixed samples of transgenic hybrids with *B. rapa* (Br) and oilseed rape (*B. napus*) (Bn). Panels A, C, and E represent hybrids mixed with *B. rapa*, and Panels B, D, and F are from hybrids mixed with oilseed rape. The arrow in each panel demarks the G1 peak (2C) of each hybrid type, and the parental type G1 peaks are labeled with Br or Bn. This series demonstrates a general reduction of ploidy as the hybrid plants are backcrossed into the *B. rapa* genetic background.

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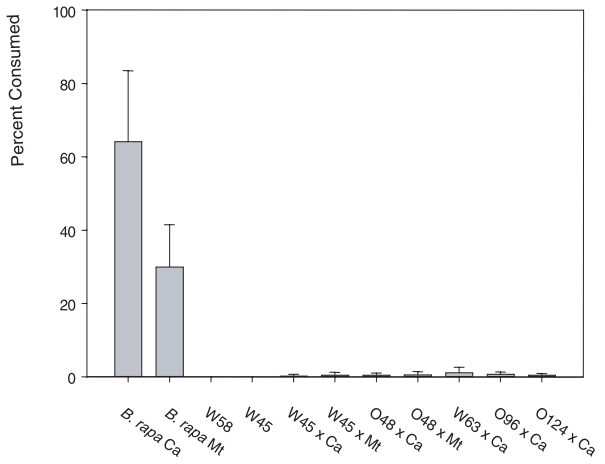

Figure 3. Average insect herbivory on several *Brassica rapa*, *Bt*-transgenic oilseed rape, and *B.rapa*-oilseed rape F_1 hybrid lines. The wild-type *B. rapa* lines suffered from heavy herbivory damage caused by *Bt*-susceptible neonate corn earworm (*Helicoverpa zea*) compared to *Bt* oilseed rape and *Bt* transgenic hybrids.

demonstrates that a small portion of the C genome (perhaps 1–2 chromosomes) was not lost in the first meiotic division. The BC_2 generation continued the trend toward the loss of genetic material, and had very similar peaks to the *B. rapa* parental line (Fig. 2). The peaks illustrated that chromosomes from the BC_1 generation seem to have been lost in the meiotic divisions in gamete formation. The ploidy level of the BC_2 generation was indistinguishable from *B. rapa*, and could represent a reduction of ploidy to a diploid number of 20 chromosomes.

Insect bioassay

Bt-susceptible neonate corn earworm (CEW) were placed on leaf disks removed from the oldest leaves of the transgenic hybrid plants, and consumption was almost completely inhibited on the hybrid plant material compared to wild type *B. rapa* (Fig. 3). Control *B. rapa* plants suffered consumption levels of 64 ± 19% in CA and 30 ± 10% in MT. Insect survivorship resulted in one large caterpillar in the control treatments, as the result of cannibalism of the larvae during the two-day period of the assay. The results of control *B. rapa* consumption contrast those of the transgenic hybrid material, in which no hybrid line suffered more than 1% consumption with no surviving insects found at the end of the assay. The insects started feeding and created a small degree of herbivory damage, but the *Bt* toxin apparently inhibited feeding and prevented greater damage. The degree of consumption of the transgenic hybrids was similar to the *Bt* parental oilseed rape lines. Despite low levels of *Bt* expression, hybrids from line W63 produced adequate Bt levels to inhibit insect herbivory. In the successive backcrosses, all material that survived the hygromycin screen also inhibited herbivory damage. The *Bt* gene was expressed in all transgenic hybrids and backcrosses at sufficiently high rates to prevent herbivory damage by CEW.

Field hybridization experiment

Under field conditions, *B. rapa* grew competitively within the oilseed rape blocks, and the wild relative flowered two weeks prior to the onset of crop flowering. Flowering occurred concurrently between the two species for several weeks. To prevent seed loss by the shattering of dry seed-pods, whole *B. rapa* plants where collected at the end of the growing season. The plants were allowed to dry completely in net bags, and seeds were collected from each of the *B. rapa* plants. Seeds were produced on 15 of the 24 *B. rapa* plants within the respective oilseed rape blocks. Hybridization was detected under field conditions and the independent lines produced hybrids at different rates (Tab. 2). O52 produced the highest hybridization rate found in this study at 16.9 ± 13%, while O96 produced the lowest rate of 0.72 ± 0.64%. Although the rate of hybridization varied within the blocks of each transgenic line, significant differences in hybridization frequency as determined by ANOVA were detected between O52 and O96 (P = 0.05, Fisher's LSD).

DISCUSSION

These results reinforce the evidence that gene flow readily occurs from transgenic oilseed rape to its weedy relative, *B. rapa* (Harper et al., 1999; Halfhill et al., 2001; Metz et al., 1997; Snow et al., 1999). Furthermore, the resultant hybrids synthesize the *Bt* Cry1Ac protein at similar levels to the parental oilseed rape lines. During successive backcrosses, the resultant transgenic plants have reduced numbers of chromosomes, and take on the morphological characteristics of the weedy *B. rapa* parent. In this case, transgenic *B. rapa*-like plants can be produced in three generations. Although two other studies have produced *Bt*-transgenic vegetable varieties of *B. rapa* using *Agrobacterium*-mediated transformation (Xiang et al., 2000; Cho et al., 2001), this is one of the

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Table 2. Production of hybrids between *Bt* oilseed rape and *B. rapa* in the field.

	F_1 Hybrids[1] (transgenic/germinated)	Rate of Hybridization (%)
B. rapa California × O96		
block a	3/343	0.8
block b	1/295	0.3
block c	6/357	1.7
block d	0/558	0.0
block e	3/355	0.8
		mean = 0.72 ± 0.65 (a)
B. rapa California × O56		
block a	35/403	8.7
block b	191/582	32.8
block c	2/537	0.3
block d	72/465	15.5
		mean = 14.33 ± 13.8 (ab)
B. rapa California × O52		
block a	51/191	26.7
block b	2/568	0.4
block c	88/487	18.1
block d	36/492	7.3
block e	77/240	32.0
		mean = 16.9 ± 13.15 (b)
B. rapa California × W58		
block a	27/380	7.1

[1] Four *Bt* oilseed rape lines were planted in 5 m^2 blocks (1 line per block) with one *Brassica rapa* plant (California) at a ratio of 1200:1. Seeds were collected from the *B. rapa* plant and germinated on Murashige and Skoog basal medium containing hygromycin (50 $mg.L^{-1}$) to select for transgenic hybrids. The rate of hybridization was calculated by comparing the number of plants that survived antibiotic selection to the total of number of germinated seedlings. a and b represent significant differences at $P = 0.05$ (Fisher's LSD).

first studies to document the gene flow of a *Bt* gene from a crop to weedy varieties of *B. rapa*. Transgene flow of the *Bt* gene has also been detected under field conditions. Although the W58 line produced no hybrids in greenhouse crosses, hybrids were generated under field conditions and demonstrate the need to reproduce hybridization experiments under agricultural conditions. The integration of transgenes into *B. rapa* seems likely to occur whenever these two species grow in close proximity, and the transgenic plants generated in this study can be used as a model system to study the ecological ramifications of transgene flow.

In agriculture, commercial releases are typically high expressing lines that aim to deliver plants with the optimal agronomic phenotype to the end user. This study illustrates that when hybridization occurs under natural conditions, the hybrid plants will have levels of expression similar to the crop lines. In another hybridization experiment, GFP (green fluorescent protein) fluorescence in transgenic hybrids was shown to

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correlate to the expression level of the oilseed rape parent, but the fluorescence intensity was similar to hemizygous parent plants (Halfhill et al., 2001). GFP translational fusions with other transgenes of interest may allow for more accurate analysis of transgene expression. The phenotype of the crop plant, be it herbicide, insect, or viral resistant, should be expected to be seen in transgenic hybrids and backcrossed progeny that occur at the borders of agriculture. Methods to mitigate this flow should be studied in order to prevent the transfer of these fitness-enhancing traits to wild populations.

The goal of this study was to track the generation of hybrid plants that could exhibit an insecticidal phenotype outside agriculture. Several factors could have underestimated the actual frequency of gene flow from the crop to weed. Antibiotic selection was used in both laboratory and field experiments to select for transgenic hybrids, and this method could have underestimated the transfer of the *Bt* transgene by killing low expressing individuals. Although low expressing primary *Bt* oilseed rape lines survived antibiotic selection, the hybrid material could have contained the *Bt* and hygromycin phosphotransferase (HPH) genes but potentially produced the recombinant proteins at lower levels than the threshold for survival. Transcriptional and post-transcriptional gene silencing could have also underestimated total gene flow. The transgene could have been present in the plant, but poor translation could have led to a false negative data point under antibiotic selection. In the laboratory crossing experiment, the population of segregating *Bt* oilseed rape plants (pollen donors) consisted of a mixture of homozygous and hemizygous plants. The hemizygous individuals could have generated non-transgenic F_1 hybrid seeds on the *B. rapa* parent from pollination events with gametes lacking the transgene through segregation. Considering these factors, total gene flow (genetic material from oilseed rape) could have been underestimated, but the emphasis of this study was to monitor the production of the insect resistance phenotype in the resultant hybrids.

The possibilities of gene flow from different independent transgenic lines of oilseed rape were investigated in this study. If certain lines of *Bt*-transgenic oilseed rape were unable to produce transgenic backcrosses, this could be an important finding to help reduce the risk of transgene escape into wild populations. It has been postulated that the location of transgene integration into oilseed rape, whether on the A or C genome, has important ramifications in the ability of the event to pass fitness-enhancing transgenes to *B. rapa* (Metz et al., 1997). This hypothesis was supported by transgenic oilseed rape lines that produced no transgenic backcrosses (Metz et al., 1997). Several sources have questioned the A versus C genome safe-spot hypothesis, and contradictory evidence suggests that the location of transgene insertion may not lead to increased biosafety in regards to gene flow (Halfhill et al., 2001; Tomiuk et al., 2000). The similarity between A and C chromosomes may lead to high rates of recombination, which could increase transgene introgression into *B. rapa* from either genome (Tomiuk et al., 2000). Halfhill et al. (2001) have hand-crossed 12 independent lines of GFP and GFP/*Bt* (GFP linked to a synthetic *Bt cry1Ac* endotoxin gene) oilseed rape with three varieties of *B. rapa*, and found that transgenic hybrids were produced at similar rates in all 36 possible combinations. If certain transgenic lines were safer in regards to gene flow, differing hybridization frequencies between the independent transformation lines would be expected. As future hybridization experiments are completed with a larger number of oilseed rape varieties and *B. rapa* ecotypes, the processes that control gene flow will be better understood. Presently, evidence suggests that certain transgenic lines may be safer in regards to gene flow, but the reason for this reduced ability may not be simply described by the location on the A or C genome.

The question of increased fitness of transgenic weeds must be addressed in future research. Non-transgenic hybrids (*B. rapa* – oilseed rape) have illustrated higher fitness under field conditions than *B. rapa* (Hauser et al., 1998a). In later studies, BC_1 plants had fitness indices similar to *B. rapa* (Hauser et al., 1998b; Snow et al., 1999). In another case, hybrids between oilseed rape and wild hoary mustard (*Hirschfeldia incana*) were shown to have a competitive advantage over wild populations of the weedy relative (Lefol et al., 1996). Fitness enhancing transgenes, such as *Bt*, could skew these advantages to a few hybrid and BC_1 individuals, and lead to the possibility of ecological consequences.

MATERIALS AND METHODS

Plant material

Seven T_3 *Bt cry1Ac* transgenic lines of oilseed rape (*Brassica napus* (L.)) cvs Oscar (O) and Westar (W), were used as pollen doners: W45, O48, O52, W58, W63, O96, and O124 (Stewart et al., 1996b). T_3 lines were generated by seed bulking homozygous and hemizygous individuals from the previous generation, and therefore each line was segregating for the presence of the

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transgene. Of these, one high expressing line (O96) had an average expression level of more than 1300 ng Cry1Ac mg^{-1} protein. Four lines (W45, O48, O52, and O124) were moderately high expressing lines (200–500 ng Cry1Ac mg^{-1} protein). Two low expressing lines (W58 and W63) had expression levels below 50 ng Cry1Ac mg^{-1} protein (Stewart et al., 1996b). Two accessions of birdseed rape (*Brassica rapa* (L.)), California (CA) (collected on the campus of the University of California at Irvine, courtesy of Art Weis) and Montana (MT) (collected in the Bitterroot Valley, courtesy of Randy Linder), were used as pollen recipients.

Growth chamber hybridization

Each transgenic oilseed rape line was germinated on MS basal media containing 50 $mg.L^{-1}$ hygromycin to select for transgenic oilseed rape plants (Murashige and Skoog, 1962), and homozygous and hemizygous individuals survived the antibiotic screen. The *Bt* oilseed rape lines were grown under high output fluorescent lights in growth chambers with a photoperiod of 16 hrs at 20/18 °C. The *B. rapa* lines were germinated two weeks later than oilseed rape on MS basal media with no antibiotics to ensure proper flowering time between the two species, and the *B. rapa* plants were moved into the same conditions as the oilseed rape lines after germination.

The self-incompatible *B. rapa* lines served as the pollen recipients. Therefore, 14 hybrid types were possible from this hybridization design. Both species were allowed to flower, and hand-crossing was performed by removing oilseed rape flowers from three plants and pollinating six *B. rapa* plants per cross. The hand-crossing continued as long as both species continued to flower. All seeds were collected from the six *B. rapa* parents in each cross and were bulked. The progeny seeds were germinated on MS basal media containing 50 $mg.L^{-1}$ hygromycin to select for transgenic hybrids. This concentration of antibiotic leads to 100% mortality of non-transgenic *B. rapa* seedlings (data not shown).

Backcrosses

Transgenic hybrids that survived the antibiotic screen were hand-crossed with the *B. rapa* parental line in the same fashion as above to produce backcrosses. The transgenic hybrids had a shorter time to flower than oilseed rape; therefore the *B. rapa* seeds were germinated at the same time to produce flowers concurrently. Two backcross generation populations (BC_1 and BC_2) were generated under these conditions.

Polymerase chain reaction (PCR)

PCR was used to confirm the presence of the transgene in the genome of the plants. Genomic DNA extraction was carried out according to Stewart et al. (1997). Specific DNA primers for a *Bt* fragment (Stewart et al., 1996a); bases 200-219 5'-ATTTGGGGAATCTTTGGTCC-3' and bases 789-770 5'-ACAGTACGGATTGGGTAGCG-3', were used to amplify the specific transgene. PCR procedure was carried out according to Stewart et al. (1996a).

Protein extraction and western blot analysis

The protein blot analysis was done according to Stewart et al. (1996b). A 0.1 N NaOH extraction buffer was added to 0.2 g of fresh plant matter. The tissue was then homogenized with a hand drill driven pestle, and incubated on ice for 30 minutes. The homogenate was neutralized with 1 M Tris-HCl pH 4.5, and centrifuged in a microfuge at 10 000 rpm for 7 minutes. The supernatant was then removed, and total protein amounts in each sample were determined by Bradford total protein analysis using BSA as a standard. For the blot, 20 µg from each sample was loaded into a 10% polyacrylamide gel along with serial dilutions of *Bt* toxin standards. Immunostaining was carried out as according to Pratt et al. (1986). For the primary antibody wash, rabbit polyclonal anti-*Bt*-toxin serum (courtesy of Dow AgroSciences) was used, followed by goat anti-rabbit (Sigma). Rabbit anti-goat/alkaline phosphatase (Sigma) was the tertiary antibody. *Bt* toxin was detected on blots by exposure to nitroblue tetrazolium/bromochloroindolyl phosphate, and the limit of detection was 2 ng *Bt* protein per lane.

Ploidy determination

Flow cytometry was used to estimate the ploidy level of all hybrids and backcrosses generated in this study. The flow cytometry was performed on a Becton Dickinson FACS Caliber flow cytometer using the side scatter monitor to analyze the data. The isolation and propidium iodide staining of nuclei was performed according to Galbraith et al. (1983). Each test sample included isolated parental nuclei as an internal control.

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Insect bioassays

Two one-inch leaf disks were removed from the oldest nonsenescing leaf on each plant, and were placed on moist filter paper in a Petri dish. Five neonate corn-ear worms (*Helicoverpa zea*) (CEW) were placed on each leaf disk. The larvae were allowed to consume the plant material for two days under a 23-hour photoperiod. Percent leaf consumption and number of live insects were recorded at the end of the two-day period.

Field experiment

Four lines of homozygous *Bt*-transgenic oilseed rape (O52, O56, W58, and O96) were planted in six 5 m^2 blocks (one line per block, 24 blocks total) with one *B. rapa* (California) plant at a ratio of 1200:1. Within the field, each 5 m^2 block was randomly planted, and the blocks were separated by a 2 m boundary on all sides. The field site was located in Tifton, GA, USA, and seeds were planted in October 1999 with a small row planter at a density of 50 plants/m^2. Four *B. rapa* seeds were planted at the center of each block, and were later culled to one wild relative plant per block. The wild relatives were planted at the same time as the oilseed rape lines, and were monitored to ensure that the wild relative and oilseed rape flowered concurrently. Seeds were harvested in May 2000 from the wild relatives only, and were germinated on MS basal media containing hygromycin (50 mg.L^{-1}) to select for transgenic hybrids. Hybridization rates were calculated by observing the number of plants that survived antibiotic selection (transgenic) compared to the total number of germinated seedlings.

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