

ORIGINAL ARTICLE

Changes in fitness-associated traits due to the stacking of transgenic glyphosate resistance and insect resistance in *Brassica napus* L.JP Londo^{1,3}, MA Bollman², CL Sagers³, EH Lee² and LS Watrud²¹National Research Council, Corvallis, OR, USA; ²US Environmental Protection Agency, National Health and Environmental Effects Research Laboratory Western Ecology Division, Corvallis, OR, USA and ³Department of Biological Sciences, University of Arkansas, Fayetteville, AR, USA

Increasingly, genetically modified crops are being developed to express multiple 'stacked' traits for different types of transgenes, for example, herbicide resistance, insect resistance, crop quality and tolerance to environmental stresses. The release of crops that express multiple traits could result in ecological changes in weedy environments if feral crop plants or hybrids formed with compatible weeds results in more competitive plants outside of agriculture. To examine the effects of combining transgenes, we developed a stacked line of canola (*Brassica napus* L.) from a segregating F₂ population that expresses both transgenic glyphosate resistance (*CP4 EPSPS*) and lepidopteran insect resistance (*Cry1Ac*). Fitness-associated traits were evaluated between this stacked genotype and five other *Brassica* genotypes in constructed mesocosm plant communities exposed to insect herbivores (*Plutella xylostella* L.) or glyphosate-drift. Vegetative biomass, seed production and

relative fecundity were all reduced in stacked trait plants when compared with non-transgenic plants in control treatments, indicating potential costs of expressing multiple transgenes without selection pressure. Although costs of the transgenes were offset by selective treatment, the stacked genotype continued to produce fewer seeds than either single transgenic line. However, the increase in fitness of the stacked genotype under selective pressure contributed to an increased number of seeds within the mesocosm community carrying unselected, hitchhiking transgenes. These results demonstrate that the stacking of these transgenes in canola results in fitness costs and benefits that are dependent on the type and strength of selection pressure, and could also contribute to changes in plant communities through hitchhiking of unselected traits. *Heredity* (2011) **107**, 328–337; doi:10.1038/hdy.2011.19; published online 23 March 2011

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Introduction

Despite ongoing ecological concern regarding the release of transgenic crops, the demand for genetically modified (GM) crops continues to increase. Primary concerns among ecologists include the potential for transgenic crops to escape from agricultural fields, to persist as weedy feral plants, and to introgress into weedy species. If transgenes provide a selective advantage in crop-weed hybrids, it is possible that higher fitness could lead to increased weediness (Chapman and Burke, 2006), such as increased fecundity of insect-resistant sunflowers (Snow *et al.*, 2003). However, constitutive expression of transgenes could also result in fitness costs when selective pressure is absent, thereby decreasing the benefit provided by resistance (Purrington and Bergelson, 1997). Understanding the potential fitness costs and benefits of combining transgenic traits in plant species is necessary to properly address both the ecological and agricultural impacts of crop production.

The majority of deregulated transgenic crops express a single 'effect' gene to convey a phenotype (for example, herbicide resistance), while relatively few have been modified to express 'stacked' (two or more) effect genes. Stacked transgenic crops can be formed by either the conventional breeding of two single transgene genotypes to produce a genotype with unlinked transgenes; or, by using standard transformation protocols, generating a stacked genotype with a transgene composed of multiple linked genes (Halpin, 2005). Recent reports suggest that stacked crops are becoming more common in US agriculture. For example, 47% of GM US corn crops and 58% of US GM cotton crops are planted as stacked varieties (USDA-NASS, 2010). As stacking transgenes becomes more common in agriculture, the potential for the escape of plants with stacked traits into weedy plant communities may increase.

Canola, *Brassica napus* L. ($2n = 38 = AACC$ genome), is an important oilseed crop species derived naturally from hybridization between *B. rapa* L. ($2n = 20 = AA$) and *B. oleracea* L. ($2n = 18 = CC$) (UN, 1935). Transgenic canola resistant to herbicides (glyphosate, glufosinate) has been widely grown in Canada and the United States since 1995 (Hall *et al.*, 2000). Since the release of GM canola, seed-mediated escape of transgenes from canola crop fields has been documented in Canada (Hall *et al.*, 2000;

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Beckie *et al.*, 2003). Migration and persistence of feral canola in non-crop environments is largely due to seed spill during harvest and transport (Knispel *et al.*, 2008; Knispel and McLachlan, 2010), and feral plants commonly contain herbicide-resistance transgenes corresponding to the preceding year's canola crop.

Pollen-mediated gene flow can also contribute to feral populations and transgene escape. *Brassica napus* is highly self-fertile, and out-crossing rates between fields or populations is low (0.2–1%) (Downey, 1999; Rieger *et al.*, 2002). Nonetheless, movement of transgenic *B. napus* pollen has been recorded up to 1–3 km from source fields (Rieger *et al.*, 2002; Devaux *et al.*, 2005), indicating long distance gene flow is possible. Volunteer and feral canola can serve as a transgene conduit between crop and weed populations by functioning as pollen sources and pollen recipients (Stewart *et al.*, 2003; Reagon and Snow, 2006). Gene flow between canola fields with different herbicide-resistant genotypes has resulted in doubly stacked, volunteer canola (Beckie *et al.*, 2003). Triply resistant plants, resistance to three different herbicides (two transgenic, one conventional mutant), have also been reported. Although stacked, double herbicide resistant plants have been identified as seed contaminants in commercial seed (Beckie *et al.*, 2003), stacked trait canola has not yet been approved for commercial release. Thus, volunteer canola populations both within fields and in weedy populations with stacked herbicide resistance suggest that gene flow between canola populations and transgene stacking can occur within a few growing seasons (Hall *et al.*, 2000, Knispel *et al.*, 2008).

Stacked transgenes could have variable fitness costs for weedy plants. Of the many plant species that have naturally evolved resistance to herbicides (as summarized in Vila-Aiub *et al.*, 2009), many demonstrate fitness costs when herbicide selection is not applied. Similarly, transgenes that are driven by constitutive promoters can result in constant production of transgenic proteins and contribute to a costly metabolic drain (Purrington and Bergelson, 1997). Despite these costs, stacked transgenes may persist through genetic hitchhiking, which occurs when selection on one engineered allele increases the frequency of a second, even when the second allele has neutral or negative fitness effects (Smith and Haigh, 1974). However, previous studies examining herbicide resistant, transgenic canola have not demonstrated increased weediness, suggesting that the escape of herbicide resistance outside crop fields may be of no consequence (Snow *et al.*, 1999; Hall *et al.*, 2000). Stacking of multiple herbicide resistance transgenes in canola appears not to greatly reduce the fitness (Simard *et al.*, 2005) and studies that have examined transgenic insect resistance (Bt) in canola × *B. rapa* hybrids (Mason *et al.*, 2003) and in wild sunflowers (Snow *et al.*, 2003) have revealed no fitness costs associated with resistance. However, the fitness effects of co-expression of these two different traits within *B. napus* remains unknown.

Given that naturally stacked, transgenic lines have been found in crop field boundaries (Hall *et al.*, 2000; Beckie *et al.*, 2003), that feral transgenic canola grows outside of cultivation (Knispel *et al.*, 2008; Knispel and McLachlan, 2010), and that there is agricultural interest in developing stacked trait varieties, it is reasonable to anticipate future occurrence of stacked traits within

ruderal and wild populations. Despite the potential for the formation of feral populations with multiple transgenes, we have little understanding of how these traits could migrate, evolve or influence native and naturalized plant communities.

The primary objectives of the experiments described below were to evaluate the fitness of stacked and single transgenic lines relative to control, non-transgenic lines in a common garden environment and to examine the potential for genetic hitchhiking under different selection pressures. We hypothesized that the expression of two different transgenes may be costly and could reduce the fitness of stacked canola relative to single trait or non-GM canola. To test this hypothesis, we developed a stacked transgenic genotype of *B. napus* expressing both glyphosate herbicide resistance and lepidopteran insect resistance as unlinked traits, as well as genotypes of each single transgene alone. Finally, we tested these plants in the presence of lepidopteran herbivores, Diamondback moth (*Plutella xylostella*) and glyphosate drift to determine the fitness costs and benefits of transgenic traits under selection.

Methods

Experimental design

Experiments were conducted over a spring–summer season (March–July) and replicated in 2 years. We confined our experiments within outdoor mesocosm greenhouses specially-designed with pollen filters to prevent the escape of all pollen or seeds from the experiment, but specifically for containing pollen and seeds of canola (Waschmann *et al.*, 2010). Each mesocosm is large enough to contain three 1.2 m² soil tubs, similar in size to small field plots, in which identical plant communities assembled from six different *Brassica* genotypes were planted. Within each tub, an 18-cell honeycomb planting grid was used to spatially balance six different *Brassica* genotypes with three replicate plants per genotype (Londo *et al.*, 2010). Eight mesocosms were planted for this experiment in each year, comprising a total of 72 plants per genotype, and a total of 432 plants for the experiment in each year.

Seeds of each of six *Brassica* genotypes were sown in greenhouses in mid-February and transplanted into the mesocosm chambers in mid-March. Plants were monitored weekly for flowering phenology. Stress treatments, either glyphosate herbicide at a drift level (0.1 × field application rate) or insect herbivores (*Plutella xylostella* eggs), were applied as detailed below, to mesocosms in early May when plants began to bolt. The weedy species *B. rapa*, the earliest genotype to bolt and flower (data not shown), was used as a temporal cue for exposure of plants to treatment application during the same developmental stage each year.

Plant material

To examine the potential for fitness costs of expressing transgenic traits, homozygous single and stacked transgenic lines of *B. napus* were required. We generated four experimental genotypes by combining two transgenic cultivars of *B. napus* that had been developed in the same genetic background (*B. napus* cv. Westar), but that express different transgene constructs (glyphosate and

insect resistance). The first GM line used was *Brassica napus* cv. RaideRR (GT73), which carries the *CP4 EPSPS* gene and *GOX* gene for resistance to the herbicide glyphosate (Monsanto, 2002). The second GM line was *B. napus* cv. TnWestar (Halfhill *et al.*, 2001) and carries the *Cry1Ac* gene for resistance to lepidopteran insect herbivory, and gene for a GFP fluorescent marker protein. Both transgene constructs were produced with *Agrobacterium* infection and previous work on hybridization and introgression of traits into *B. rapa* demonstrate that both transgene constructs are present in the A genome of *B. napus* (Monsanto, 2002; Mason *et al.*, 2003). The crossing scheme used to combine both GM traits from the parental lines and ensure homozygosity in the offspring is illustrated in Figure 1.

Breeding design

Reciprocal F_1 crosses were made between RaideRR and TnWestar parental lines. F_1 crosses were expected to produce 100% glyphosate resistant and 100% insect resistant progeny because of the homozygous dominant state of each transgene construct in the parental lines. Progeny of the F_1 cross were screened for glyphosate

resistance by application of glyphosate spray ($2 \times$, 4.681 ha^{-1}), followed by a quality check and verification with lateral flow TraitChek strips (SDIX, Newark, DE, USA). Progeny were screened for insect resistance by visualizing the expression of the linked GFP marker with a Spectroline BIB-150P UV Lamp (Halfhill *et al.*, 2001), followed by quality check and verification with Ecologix Quickstix strips (Ecologix, Portland, ME, USA). All progeny of the F_1 cross were both glyphosate resistant and insect resistant, affirming the homozygous state of the original parent lines (J Londo, personal observation). A single F_1 hybrid plant from the RaideRR \times TnWestar family served as the paternal parent in F_2 crosses with a F_1 hybrid plant from a reciprocal TnWestar \times RaideRR family (Figure 1). F_2 seeds (50) were planted and screened for both traits. Segregation of the nine possible genotypes was not significantly different from expected values for two unlinked traits (χ^2 , $P = 0.57$).

Homozygous F_2 plants for stacked transgenic, single transgenic and non-transgenic genotypes were identified by screening F_3 progeny produced from manual crosses between F_2 pollen donor plants and non-transgenic *B. napus* cv. Westar (Figure 1). All F_2 plants were also self-fertilized and bagged to prevent cross pollination. The four *B. napus* lines will henceforth be described based on their transgenic genotype as Null (no transgene expression), RR (glyphosate resistant), BB (Bt insect resistant) and RRBB (stacked-glyphosate and insect resistant). Null *B. napus* plants represent a good non-transgenic comparison for evaluating the cost of multiple transgenes relative to single transgenes, and also to minimize the potential for fitness differences (for example, inbreeding depression, heterosis) resulting from the breeding scheme. All seedlings used in the experiment for the four *B. napus* lines were assessed for transgene expression using lateral flow test strips that detect the production of transgenic proteins for each transgene construct (*CP4 EPSPS*—TraitChek, Strategic Diagnostics, Newark, DE, USA; *Cry1Ac*—QuickStix strips, EnviroLogix, Portland, ME, USA), before transplantation into mesocosms.

The mesocosm plant community was composed of the four different genotypes of *B. napus* described above. In addition, one genotype of each of the weedy species *B. rapa* and *B. nigra* were included to simulate competition from other sexually compatible brassicaceous weeds found at field boundaries. The *B. rapa* genotype used was collected from a local weedy population in Corvallis, OR, USA. A local population of *B. nigra* was not available, thus the *B. nigra* genotype used was obtained from the USDA GRIN germplasm center (accession no. PI 649154).

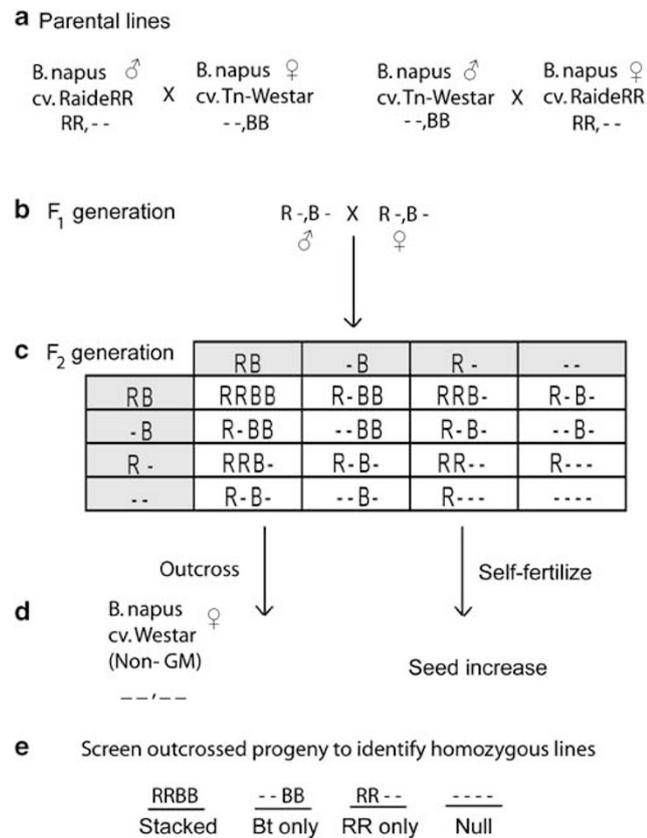


Figure 1 Breeding scheme for development of stacked transgenic glyphosate resistant/Bt insect resistant *Brassica napus*. (a) Parental lines for glyphosate tolerance (RR) and insect tolerance (BB) were reciprocally crossed to produce (b) F_1 generation plants. (c) F_1 generation was crossed to produce a F_2 segregating population. (d) Each F_2 plant was manually out-crossed to non-GM *B. napus* cv. Westar and self-fertilized to increase seed numbers of each genotype. (e) F_3 seeds from out-crossed *B. napus* are screened to identify plant lines that carry the transgenes as homozygous genes.

Treatments

Replicate communities were constructed in eight mesocosms in each of 2 years. Three mesocosms were maintained as no-herbivore, no-glyphosate controls. We applied two different selective pressures, insect herbivory and glyphosate herbicide, to the remaining mesocosms to test the potential fitness benefits of herbivore resistance and glyphosate resistance transgenes. Though no treatment carryover was expected between mesocosms between the two years, control and selective treatments were randomly assigned to mesocosms between the 2 years of the study.

Plutella xylostella (Diamondback moth) is a specialist lepidopteran pest of *Brassicaceae* species. Previous data for infestations of *Brassica* species by diamondback moth demonstrate a range of 1–100 larvae per plant (Bigger and Fox, 1997). We simulated a moderate to heavy infestation of herbivores of ~50–75 larvae per plant. Observations of hatching of eggs in Petri-dish experiments demonstrated ~30–50% of commercially available (Bio-Serv, Frenchtown, NJ, USA) eggs successfully hatched (personal observation). Insect eggs were introduced to the host plants by placing small paper squares containing 50–75 eggs each at two locations on each plant, one at a leaf axil at mid-height and the second on one of the lowest leaf axils. The herbivory treatment was applied to three of the eight mesocosms in each of the 2 years of the study.

A second selective treatment was a single application of glyphosate at a concentration of $0.1 \times$ the rate (2.341 ha^{-1}) that would be typically applied to crop canola. This treatment was designed to simulate a non-target rate of glyphosate exposure, such that might occur near fields or roadsides as a result of aerial drift (Yates *et al.*, 1978; Ganzelmeier and Rautmann, 2000; Bird *et al.*, 2002). Timing of the glyphosate treatment was not intended to simulate glyphosate applications within a canola field, but in less managed areas where drift could occur at different times depending on the adjacent crop (for example, corn, soybean) or weed management target, such as along transportation routes. Glyphosate was applied to each mesocosm using an even flat fan spray nozzle (TeeJet 80015VS, Spraying Systems Co., Wheaton, IL, USA) held 45.7 cm above the plant canopy. Glyphosate drift treatment was applied to two of the eight mesocosms in each of the 2 years.

Brassica napus is predominantly self-fertile; however, it can be pollinated by both wind and insects. In contrast, both weedy species, *B. rapa* and *B. nigra*, are highly self-incompatible and require pollinators for seed production. One thousand generalist pollinators (*Musca domestica* L.) (Rincon-Vitova Insectories, Ventura, CA, USA) were added to each mesocosm 5 days after selective treatments were applied (following Halfhill *et al.*, 2003). Pollinators remained active throughout the course of the experiment in all mesocosms until harvest, at which time insect populations were terminated using electronic insect control (Flowtron Insect Killer, Malden, MA, USA).

Data collection and analysis

The above-ground biomass of all plants was harvested by hand to prevent loss of seeds due to seed shattering at the end of the growing season (120 days). Each mesocosm was harvested to completion before progressing to additional mesocosms. Harvest was concluded within a week and seed maturation rates were comparable between mesocosms and among treatments. Vegetative biomass (VBM) and seed production were used as proxies of plant fitness (Simard *et al.*, 2005). VBM, stems, leaves and siliques, VBM and seed biomass (SBM) were recorded for each individual. Stems and leaves were dried for 10 days in a forced air oven at 60°C . *Brassica* siliques were collected from each individual in separate envelopes and dried at ambient temperatures for a minimum of 14 days. Following drying, seeds were removed from siliques, weighed and recorded as total

SBM. Several response variables were derived from the vegetative and SBM measurements to evaluate different aspects of plant fitness. Traits included: total VBM (TVBM), weight of stems, leaves and siliques; estimated seed number (ESN) determined from individual seed weights from 100-seed sub samples for each plant, multiplied by SBM; and relative seed production (RSP), the number of seeds produced per gram of vegetative tissue (ESN/TVBM) for each individual plant. Measures of the costs of sensitivity and benefits of resistance for each genotype were calculated by taking the mean number of seeds produced by each genotype in each treatment, divided by mean seed production of each genotype in control mesocosms. Measures of relative fecundity (sic Spencer and Snow, 2001; Chen *et al.*, 2006) were calculated by taking the mean number of seeds produced by transgenic genotypes, divided by the mean number of seeds produced by the Null genotype. Thus, this measure indicates the observed cost or benefit of having different transgenes in the different mesocosm treatments as a function of the seed production of the non-transgenic, sensitive, genotype.

Fitness-associated traits were measured from individual plants and measurements were tested for a normal distribution. Genotypes within mesocosm tubs are not independent replicates and data was analyzed by multivariate analysis of variance with genotype as a within-subject factor. The main effects of treatment type, year and their interactions were tested at the mesocosm level using the whole plot error term. When interactions between main effects were significant ($P < 0.05$), subsequent analyses were performed on subsets of the data (Snedecor and Cochran, 1980). Although unequal sample sizes can influence the rate of type II errors, analysis of variance is robust to modest departures from unequal sample sizes, as is the case in this experiment, and no additional steps were needed to conduct the analysis. Statistical analyses were generated using SAS/STAT software (SAS Institute Inc., Cary, NC, USA) (PROC GLM), Version 9.1 of the SAS system for Windows.

To evaluate fitness-associated traits in control mesocosms without selection, *post-hoc* contrasts were conducted between stacked RRBB, and the Null and single transgenic *B. napus* genotypes. To correct for error due to multiple testing, we used a sequential Bonferroni correction to compare these contrasts and adjust *P*-values if necessary (Rice, 1989).

Results

Multivariate analysis revealed a significant genotype by glyphosate/herbivore-treatment term, indicating that genotypes responded differently to treatment for all plant responses ($P < 0.001$) (Supplementary Table S1). A significant main effect of treatment, and specifically glyphosate-drift treatment, was also evident for ESN ($P = 0.045$) and RSP ($P < 0.001$) values (Supplementary Table S1). Neither herbivory nor glyphosate treatment affected TVBM; however, year had a significant effect on TVBM ($P < 0.001$) (Supplementary Table S1). No interaction was observed between treatment type and year. Total community biomass was not significantly different between treatment types in either year as biomass loss of sensitive genotypes was balanced by biomass gain of resistant genotypes (data not shown). Because of the

Table 1 Results of ANOVA for plant response variables from genotype datasets

	TVBM	ESN	RSP
<i>Null</i>			
Herbivory	0.786	0.085	0.007
Glyphosate	0.273	< 0.001	< 0.001
Year	0.001	0.002	0.209
Herb × Y	0.792	0.863	0.850
Gly × Y	0.638	0.551	0.731
<i>RR</i>			
Herbivory	0.083	0.804	0.054
Glyphosate	0.013	< 0.001	0.004
Year	0.138	0.644	0.158
Herb × Y	0.662	0.843	0.734
Gly × Y	0.042	0.113	0.282
<i>BB</i>			
Herbivory	0.372	0.618	0.847
Glyphosate	0.196	0.001	< 0.001
Year	0.005	0.182	0.620
Herb × Y	0.761	0.552	0.411
Gly × Y	0.392	0.489	0.088
<i>RRBB</i>			
Herbivory	0.004	0.012	0.557
Glyphosate	< 0.001	< 0.001	0.330
Year	0.152	0.017	0.552
Herb × Y	0.812	0.132	0.658
Gly × Y	0.657	0.709	0.254
<i>B. rapa</i>			
Herbivory	0.029	0.577	0.806
Glyphosate	0.002	0.005	0.009
Year	< 0.001	0.074	0.602
Herb × Y	0.019	0.351	0.662
Gly × Y	0.375	0.769	0.887
<i>B. nigra</i>			
Herbivory	0.687	0.325	0.896
Glyphosate	0.951	0.156	0.081
Year	0.403	0.963	0.727
Herb × Y	0.330	0.823	0.677
Gly × Y	0.937	0.768	0.374

Abbreviations: ANOVA, analysis of variance; ESN, estimated seed number; RSP, relative seed production; TVBM-Total vegetative biomass.

Brassica napus genotypes denoted by transgene state. Plant fitness-associated traits measured: TVBM, ESN, RSP. Effects tested include main effects of the two treatment types (Herbivory, Glyphosate) and Year, as well as interaction effects (Herb × Y, Gly × Y) for each treatment in each year. Values represent *P*-values generated by statistical analysis. Significant values indicated in boldface type for $P < 0.05$.

significant interaction of genotype and treatment, subsequent statistical analyses were performed on genotype and treatment datasets independently. Analysis of the data by genotype demonstrated significant effects of treatment, year, and in some cases, the interaction between these effects (Table 1). Overall, plants of all genotypes were larger and produced more seeds in the second year of the study (data not shown).

Fitness difference among plant genotypes

Comparisons between the *B. napus* genotypes in control mesocosms indicated that the fitness of single-transgene genotypes did not significantly differ from the Null

genotype. However, in the absence of selective pressure, stacked RRBB plants produced the smallest VBM, the fewest seeds, and the lowest RSP of the four *B. napus* genotypes. Compared with the Null genotype, RRBB plants produced significantly less VBM ($P = 0.020$), lower ESN ($P = 0.005$) and lower RSP values ($P = 0.017$). Relative to the single-transgene RR genotype, RRBB plants produced significantly less TVBM ($P = 0.011$), lower ESN ($P = 0.004$) and lower RSP ($P = 0.008$). Contrasts between the stacked genotype and the Null and RR genotypes remained significant after analysis with sequential Bonferroni tests. Relative to the single-transgene BB genotype, RRBB plants tended to be smaller and less productive, but these differences were not significant (Figure 2). Among the six plant types, *B. rapa* produced the greatest VBM and had seed production comparable with *B. napus*. *Brassica nigra* plants produced the least VBM and the fewest seeds.

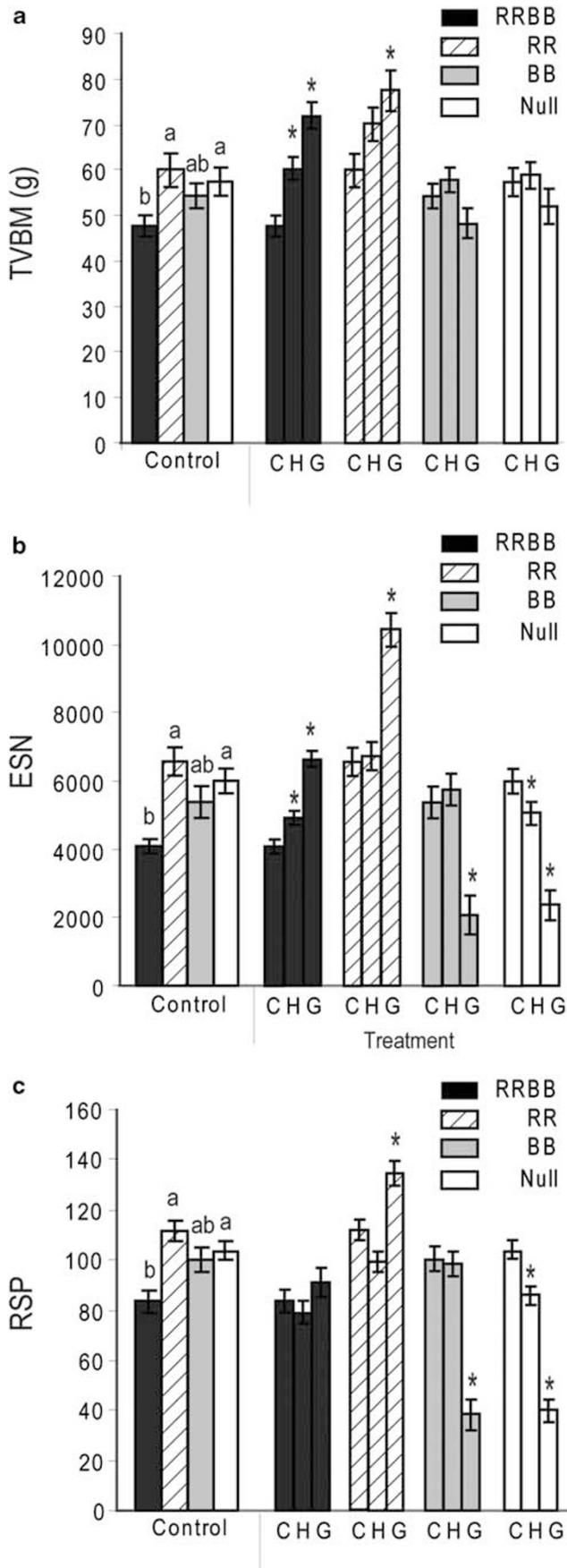
Herbivory pressure

Despite comparable herbivore resistance, for example, we observed no herbivore damage on BB or RRBB plants, the fitness benefit of insect resistance differed between the resistant genotypes. VBM and seed production of the BB genotype were both greater under herbivory selection pressure than under control conditions, though not significantly (Table 2 and Figure 3a). In contrast, the stacked RRBB genotype was significantly larger (26%), and produced significantly more seeds (20%) in the presence of herbivores (Table 2 and Figure 3a). Despite these increases, the RRBB genotype did not out-perform the BB genotype under selection, producing 14% fewer seeds and the lowest RSP of the *B. napus* genotypes.

Damage from larval diamondback moths was observed throughout the course of the experiment; first as leaf mining and damage to leaves, and later as active herbivory on flowers and developing siliques of susceptible genotypes. However, though the level of herbivory used in this experiment was moderately-high (Bigger and Fox, 1997), herbivore damage did not have strong effects on the fitness traits measured. Null *B. napus* plants produced 16% fewer seeds and had significantly reduced RSP because of herbivory (Table 2 and Figure 3a). The number of seeds produced and TVBM did not decrease for RR plants, though a trend of lower RSP values ($P = 0.054$) were observed (Table 2). Only the weedy *B. rapa* genotype had a significant reduction in TVBM with herbivory.

Glyphosate-drift pressure

Exposure to glyphosate-drift had no negative effect on the glyphosate resistant RRBB and RR plants. With glyphosate-drift treatments, RRBB plants produced significantly greater VBM (49% greater) and increased seed numbers (62% more) relative to control mesocosms (Table 2 and Figure 3a). RR plants also produced significantly higher VBM (29%), greater seed numbers (58% more) and had significantly higher RSP with the glyphosate-drift treatment as compared with control mesocosms (Table 2 and Figure 3a). Moreover, the RR genotype had the highest fitness of all the genotypes in glyphosate-drift mesocosms for all measured traits. The glyphosate-drift treatment was a much stronger selective force than herbivores on the sensitive genotypes in the



mesocosm communities. All of the sensitive genotypes in the community had significant reductions in reproductive measures (Table 2 and Figure 2). In contrast, VBM measurements were not as indicative of glyphosate-sensitivity and were only significantly reduced for *B. rapa*.

Relative costs and benefits of transgenic resistance

To assess the costs of single and multiple transgenic resistance traits in *B. napus*, the relative fecundity of the transgenic genotypes was examined by comparing the number of seeds produced by transgenic genotypes to the seeds produced by the Null genotype. In control mesocosms, the stacked RRBB genotype had the lowest relative fecundity, producing significantly fewer seeds, -32%, than the Null genotype (Table 2 and Figure 3b). The BB genotype also had lower relative fecundity than

Table 2 Plant response measurements for the six *Brassica* genotypes

	TVBM (g)	ESN (seed #)	RSP (seeds g ⁻¹)
<i>Null</i>			
Control	57.6 ± 3.1	5998 ± 350	103.8 ± 3.7
Herbivory	58.8 ± 3.1	5051 ± 350	86.0 ± 3.7*
Glyphosate	51.9 ± 3.8	2364 ± 435*	39.8 ± 4.6*
<i>RR</i>			
Control	60.0 ± 3.7	6581 ± 401	111.4 ± 4.1
Herbivory	70.1 ± 3.7	6724 ± 393	99.0 ± 4.0
Glyphosate	77.5 ± 4.5*	10428 ± 481*	137.7 ± 4.9*
<i>BB</i>			
Control	54.3 ± 2.8	5397 ± 461	99.8 ± 5.1
Herbivory	59.0 ± 2.7	5731 ± 456	98.4 ± 5.0
Glyphosate	48.3 ± 3.4	2057 ± 476*	38.2 ± 6.3*
<i>RRBB</i>			
Control	47.8 ± 2.4	4082 ± 191	83.3 ± 4.9
Herbivory	60.3 ± 2.4*	4907 ± 191*	79.1 ± 4.9
Glyphosate	71.2 ± 2.9*	6638 ± 234*	91.2 ± 5.9
<i>B. rapa</i>			
Control	80.4 ± 2.4	4533 ± 575	59.5 ± 7.9
Herbivory	72.0 ± 2.3*	4066 ± 569	56.7 ± 7.8
Glyphosate	62.9 ± 3.6*	800 ± 881*	12.3 ± 12.1*
<i>B. nigra</i>			
Control	43.2 ± 4.8	1912 ± 385	45.3 ± 7.1
Herbivory	46.3 ± 5.5	2515 ± 438	54.3 ± 8.0
Glyphosate	42.5 ± 10.2	536 ± 810	13.4 ± 14.9

Abbreviations: ESN, estimated seed number; RSP, relative seed production; TVBM—Total vegetative biomass.

Plant response variables include: TVBM, ESN and RSP. Results presented as means ± one standard error. Means followed by an asterisk indicate significant treatment difference from control at *P* < 0.05.

Figure 2 *Brassica napus* genotype fitness-associated traits. For genotype contrasts in control, shared letter indicates no significant difference in control treatments. Error bars represent means ± one standard error. Asterisks indicate effects of treatment on genotype plant responses are significantly different from control responses at *P* < 0.05. (a) TVBM—Total vegetative biomass. (b) ESN—Estimated seed number. (c) RSP—Relative seed production. Treatments denoted on x axis for control (C), herbivory (H) and glyphosate-drift (G).

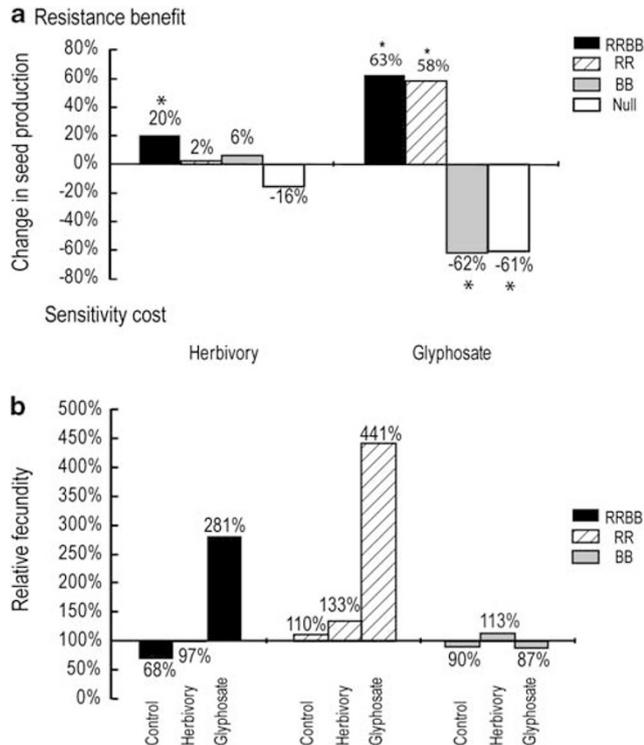


Figure 3 Seed production costs and benefits due to transgenes and effects of selection pressures. (a) Change in seed production of each *B. napus* genotype in herbivory and glyphosate-drift mesocosms relative to seeds produced in control mesocosms. Positive percent values indicate an increase in seed production under selection, negative values represent a decrease of seed production under selection. Asterisk indicates significant changes in seeds produced from control mesocosms at $P < 0.05$. (b) Relative fecundity measures for transgenic genotypes. Percent values indicate the mean seed production of each transgenic genotype, relative to the mean seed production of the non-transgenic Null genotype. Values $< 100\%$ indicate production of less seeds than the Null, non-transgenic genotype.

the Null genotype and produced 10% fewer seeds. In contrast, the RR genotype had 10% greater relative fecundity than the Null genotype in control mesocosms. Neither the 10% decrease for the BB genotype, nor the 10% increase for the RR genotype was significant (Table 2 and Figure 3b). These results indicate that without selection pressure, there are reproductive fitness costs due to the expression of two transgenes together, but not for each transgene alone.

Measures of relative fecundity in herbivory treatments were affected by changes in the seed production of transgenic genotypes, and also by seed decreases in the Null genotype due to herbivore sensitivity. In herbivory treatments, the resistant RRBB and BB genotypes and the sensitive RR genotype each increased in relative fecundity by 29, 23 and 23%, respectively (Figure 3b). All three increases are due in part, to decreases in the seed production of the herbivore sensitive Null genotype. Only the RRBB genotype had a significant increase in a seeds produced, 20% greater, (Figure 3a) that corresponded to increased relative fecundity. Transgenic herbivore resistance in herbivory mesocosms thus partially alleviated the cost of stacked transgenes observed in control mesocosms. The relative fecundity

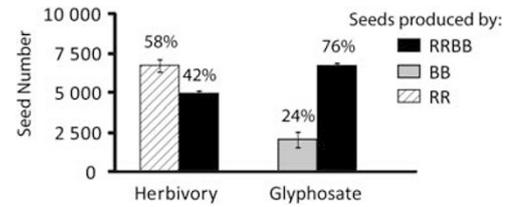


Figure 4 Seed production of the unselected transgenic traits in single and stacked transgenic genotypes in the two selective pressures. Bars indicate production of seeds (estimated) that are produced with an unselected transgene by the transgenic genotypes in the two selective treatments. In herbivory, 58% of seeds with an unselected transgene are produced by RR genotypes and 42% are produced by stacked RRBB genotypes. In glyphosate treatments, 24% of seeds with unselected transgenes are produced by the BB genotype while 76% of seeds are produced by the stacked genotype. Hitchhiking of unselected transgenes as stacked progeny rather than as single transgene progeny is much greater (320%) in glyphosate-drift treatments than in herbivory treatments (73%). Error bars indicate \pm one standard error measurement.

increases observed for RR and BB genotypes were not significant (Table 2) from the fecundity of these genotypes in control mesocosms. This result indicates that there was not a significant benefit for the BB transgene alone and under herbivory selection, or a detectable cost of expressing the RR transgene without glyphosate pressure.

Similar to herbivory treatments, measures of relative fecundity in glyphosate-drift treatments were affected both by changes in the seed production of transgenic genotypes, and the seed decreases in the Null genotype because of glyphosate sensitivity. In glyphosate-drift treatments, the resistant RRBB and RR genotypes demonstrated a strong benefit of glyphosate resistance and significantly increased seed production compared with control mesocosms by 63 and 58%, respectively (Table 2 and Figure 3a). Glyphosate resistance of the RR genotype contributed to a relative fecundity of 441% of the Null genotype, an increase of 331% over control mesocosm levels (Figure 3b). Similarly, the RRBB genotype benefitted from glyphosate resistance and increased by 313% over control mesocosm levels to a relative fecundity to 281% of the Null genotype (Table 2 and Figure 3b). Glyphosate-drift selection resulted in a significant decrease in seeds (-62%) produced by the sensitive BB genotype compared with control mesocosms (Table 2 and Figure 3a). As both the BB and Null genotypes suffered from significant decreases in seed production, the relative fecundity of the BB genotype (87%) did not drastically change from the difference observed in control mesocosms (90%) (Figure 3b).

Transgene hitchhiking

Despite the costs observed for the RRBB genotype in control mesocosms, selection by herbivores and glyphosate-drift resulted in transgene hitchhiking within the mesocosm communities. Herbivory resistance in herbivore mesocosms contributed to a significant increase in seeds produced by the RRBB genotype. Consequently, seed production by RRBB plants increased the number of seeds in the population carrying the RR transgene by 4907 (Figure 4), without selection for this trait. Similarly, strong selection for the RR transgene in glyphosate-drift

mesocosms significantly increased the number of seeds produced by the RRBB genotype. This fitness increase resulted in a greater number of seeds with insect resistance, 6638 seeds per plant ($3.2 \times$ the number of seeds produced by the BB plants) without selection for the BB transgene.

Discussion

We conducted this study to examine the effects of two environmental stresses on the relative fitness of different transgenic genotypes of *B. napus*, and to evaluate the potential for ecological effects of transgene stacking outside of crop fields. Previous studies have failed to detect fitness costs of singly expressed herbicide resistance or insect resistance transgenes in *Brassica* (Snow *et al.*, 1999; Mason *et al.*, 2003). In addition, fitness costs have not been observed when different herbicide resistance transgenes are stacked (Simard *et al.*, 2005) observed in volunteer and feral *B. napus* plants in Canadian cropping systems (Hall *et al.*, 2000; Beckie *et al.*, 2003; Knispel *et al.*, 2008). If seed production is an adequate estimator of plant fitness for an annual plant, then single transgenic and Null genotypes of *B. napus* in this study were equally fit in control mesocosms while the stacked transgenic line demonstrated fitness costs and produced the least number of seeds.

Under the different selective pressures, costs or benefits of transgenic resistance were observed. We detected a weak, but non-significant cost associated with the expression of the BB transgene alone without selective pressure. Under herbivore selection, this cost was alleviated as BB plants did not have reduced fitness measures, but no benefit or fitness increase was observed. In contrast, no reproductive cost was observed for the RR transgene when it was expressed without selection. In addition, the fecundity of RR plants was not reduced by herbivory treatment. This result might suggest that the herbivore selection in this experiment was not intense enough to result in fitness decreases on sensitive genotypes. However, herbivory did reduce seed production of the Null genotype and *B. rapa*, arguing that herbivory was sufficient to limit seed production on sensitive genotypes. In addition, the increase in relative fecundity of the RR genotype in herbivory mesocosms was actually due in large part, not to a significant increase in seeds produced by RR plants, but by the trend ($P = 0.085$) of decreasing fecundity of Null plants. The results for single transgenic genotypes in this study are thus in agreement with previous studies that have failed to detect fitness costs for each of the original parental genotypes; *B. napus* cv. RaideRR (Monsanto, 2002) and *B. napus* cv. TnWestar (Mason *et al.*, 2003), and demonstrate that the costs associated with expressing either single transgene in the absence of selection are weak (BB) or undetectable (RR).

If the costs of each transgene were additive, the stacked genotype plants would not be expected to demonstrate fitness costs in control mesocosms, at least no greater costs than the slightly lower seed production of BB plants (relative to Null plants). However, the stacked genotype was significantly smaller, produced fewer total seeds and was the least efficient at producing seeds (RSP). Regardless of the type of selective pressure, RRBB plants had lower reproductive fitness than all

other resistant plants in the mesocosm communities. These observations demonstrate that while neither transgene alone confers a substantial fitness cost, when the traits are combined, the stacked genotype is less fit than either of the single transgene or Null genotypes. Thus, the combined cost of BB and RR transgene expression is not additive, but in excess of each transgene alone. In addition, while transgenic resistance to selection pressure contributed to increased reproductive fitness in RRBB plants, the original cost could not be overcome under selection pressure. In a similar study, Chen *et al.* (2006) examined single and stacked transgenic insect resistant rice (*Oryza sativa* L.) under varied herbivory pressure. Stacked plants had lower fitness, though only under competition with mixed plant species. Future studies should include alterations in plant density and mixed species compositions with stacked trait canola to determine if competition increases costs and decreases population level persistence of feral transgenic canola, or if the fitness increases we observed because of selection are maintained despite competition.

Despite overall lowered reproductive fitness due to stacking, the stacking of transgenes in the RRBB genotype resulted in genetic hitchhiking in the mesocosm communities. Hitchhiking effects were greater in glyphosate-drift mesocosms where strong selection pressure simultaneously reduced the number of seeds produced by the BB genotype, but also contributed to a significant fitness increase of the RRBB genotype. Consequently, the total number of seeds carrying the B transgene in the population increased. A similar, but smaller effect occurred for the R transgene under herbivory selection. As a result, increased spread of transgenic seeds with neutral or unselected transgenes could occur in weedy environments similar to our mesocosm communities, if either type of selective pressure were applied. However, our results suggest that not all selection pressures are equivalent and the strength of selection pressure could greatly influence the rate of hitchhiking and transgene stacking. Genetic hitchhiking has been implicated in the spread and persistence of detrimental alleles in plant populations (Smith and Haigh, 1974; Barton, 2000) and also as a potential mechanism to contain and limit transgene escape (Rose *et al.*, 2009). In this study, the two transgenes were not physically linked, but each selective pressure acted to increase the number of seeds carrying unselected resistance alleles at the population level.

Testing for fitness costs associated with naturally evolved herbicide resistance or resistance conferred by transgenes can be complicated by differences in genetic background between plant populations (Vila-Aiub *et al.*, 2009) and degree of selection during cultivar development (Raymer and Grey, 2003). To account for these difficulties, the different transgenic genotypes evaluated in this study were developed from a restricted genetic pool to distinguish fitness characteristics primarily due to transgene expression and not to heterosis. As such, this experiment serves as a case study of the potential tradeoffs and consequences of different transgenes and different stresses. Transgene stacking in nature would likely involve the blending of different crop cultivar and weed genetic backgrounds. The combination of genetic background and variation in environmental stress could potentially compound the fitness effects of multiple

transgenes with that of the vigor often associated with hybridization (Riaz *et al.*, 2001; Rose *et al.*, 2009). Future field experiments should be conducted to test the validity of our observations and to account for the potential variation from other transgenes, crop cultivars and species. In addition, it would be of interest to examine the effects of plant population density, and other biotic and abiotic stresses alone and in combination on transgene costs.

Different hypotheses have been presented to explain the reduced fitness and costs of transgene expression in plants, including the toxic buildup of constitutively expressed proteins, increased metabolic load because of production of extra proteins (Purrington and Bergelson, 1997), and linkage to detrimental crop alleles (Halfhill *et al.*, 2005). Similarly, natural evolutionary adaptation of plants to herbicides and the development of resistance frequently results in fitness costs due to shifts in metabolism and to pleiotropic effects (Vila-Aiub *et al.*, 2009). As the specific proteins produced by the transgenes in this study have not been shown to be toxic (Monsanto, 2002; Mason *et al.*, 2003), it is perhaps more likely that metabolic load may be contributing to the costs observed. It is also possible that the fitness costs observed in this study may not result solely from the expression of the CP4 EPSPS-GOX or *Cry1Ac* transgenes alone, but may be due, in part, to constitutive expression of the linked marker genes (for example, *Cry1Ac-GFP*) or to pleiotropic effects of linked genomic DNA associated with each transgene insert (Stewart *et al.*, 2003; Chapman and Burke, 2006). The evidence for seed production costs in stacked plants in conditions that lack selection pressure suggests that the long-term persistence of stacked transgenic plants in the wild may be limited. Results from this experiment suggest that there would be a substantial fitness benefit for the loss of either transgene, but particularly the B transgene. Similar to the production of metabolically expensive defense compounds or naturally evolved herbicide resistance, constant selection pressure would be needed to counter fitness costs and contribute to continued presence of stacked transgenic plants in weedy environments.

In conclusion, our results indicate the potential for individual plant fitness costs, but also population level effects for unselected transgenes associated with the stacking of the CP4 EPSPS-GOX and *Cry1Ac-GFP* constructs within *B. napus*. As expected, each resistant genotype was more reproductively fit when challenged by the specific selective pressure, while sensitive genotypes had reductions in fitness-associated traits or were unaffected. Measures of seed production and relative fecundity demonstrated that the cost of stacked transgenes was in excess of costs observed for each transgene alone. The cost of stacked transgenes was partially alleviated under selective pressure by the benefit of herbivory resistance and also glyphosate resistance. However, despite costs in control mesocosms and underperformance in each selective treatment, the RRBB genotype produced thousands of seeds in herbivory and glyphosate-drift mesocosms. Transgenic hitchhiking thus resulted in the production of a greater number of transgenic resistant seeds in the population for future volunteer generations without selection pressure.

Conflict of interest

The authors declare no conflict of interest.

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