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GENETIC TRANSFORMATION AND HYBRIDIZATION

Characterization of directly transformed weedy *Brassica rapa* and introgressed *B. rapa* with Bt *cry1Ac* and *gfp* genes

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Abstract Crop to weed transgene flow, which could result in more competitive weed populations, is an agricultural biosafety concern. Crop *Brassica napus* to weedy *Brassica rapa* hybridization has been extensively characterized to better understand the transgene flow and its consequences. In this study, weedy accessions of *B. rapa* were transformed with *Bacillus thuringiensis* (Bt) *cry1Ac*- and green fluorescence protein (*gfp*)-coding transgenes using *Agrobacterium* to assess ecological performance of the wild biotype relative to introgressed hybrids in which the transgenic parent was the crop. Regenerated transgenic *B. rapa* events were characterized by progeny analysis, Bt protein enzyme-linked immunosorbent assay (ELISA), Southern blot analysis, and GFP expression assay. GFP expression level and Bt protein concentration were significantly different between independent transgenic *B. rapa* events. Similar reproductive productivity was observed in comparison between transgenic *B. rapa* events and *B. rapa* × *B. napus* introgressed hybrids in greenhouse and field experiments. In the greenhouse, Bt transgenic plants experienced significantly less herbivory damage from the diamondback moth (*Plutella xylostella*). No differences were found in the field experiment under ambient, low, herbivore pressure. Directly transformed transgenic *B. rapa* plants should be a helpful experimental control to better understand crop genetic load in introgressed transgenic weeds.

Keywords Crop–weed hybridization · Transgene introgression · Genetic load · Biotechnology risks · Gene flow · Field mustard

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Introduction

While transgenic plants have been grown commercially for more than 10 years, there is continued debate about their risks and regulations (Stewart 2004). Gene flow remains a major focus since some level of transgene movement from commercial transgenic crops is likely to be inevitable (Timmons et al. 1996). *Brassica napus* or oilseed rape, OSR, will certainly participate in interspecific gene flow, especially to *B. rapa* (Stewart et al. 2003; Warwick et al. 2003; Legere 2005), which might lead to the creation of more competitive and invasive hybrid populations (Mikkelsen et al. 1996; Stewart et al. 2003; Al-Ahmad et al. 2004). Hybridization between transgenic OSR and wild *B. rapa* has been confirmed by the presence of crop-specific markers and transgenes in hybrid populations (Warwick et al. 2003; Legere 2005). In field experiments, the flow of the insect resistant transgene *Bacillus thurigiensis* (Bt) *cry1Ac* from transgenic OSR to wild relatives occurred under varied field conditions (Halfhill et al. 2004). The fitness of some *Brassica* crop-weed hybrids has been shown to be different when compared to their parents (Hauser et al. 1998; Snow et al. 1998; Halfhill et al. 2005).

To date, many researchers have attempted to determine the consequences of hybridization and introgression between transgenic crops and their wild relatives (Snow and Moran-Palma 1997; Gueritaine et al. 2002; Chevre et al. 2003; Halfhill et al. 2005; Legere 2005), but little is known about the persistence or ecological effects of transgenes and linked crop genes that enter wild populations through pollen movement (Snow and Moran-Palma 1997). Halfhill et al. (2005) reported that crop–weed hybrids, with or without transgene introgression, had lower fitness and competitive ability than their parent populations, suggesting that fitness depression of crop–weed hybrids may be caused by crop genes (genetic load) in hybrids or factors other than the presence of the transgene itself. Bt *cry1Ac* expression has no measurable fitness penalty in OSR (Mason et al. 2003), and GFP is also an ecologically neutral transgene (Harper et al. 1999; Stewart 2006).

The purpose of this study was several fold. First, in order to control for genetic background in *B. napus–B. rapa* hybrids in introgression experiments, we produced directly transgenic *B. rapa* events with transgene constructs identical to those introgressed from crop to weed: constitutively expressed Bt *cry1Ac* and *gfp* transgenes via an *Agrobacterium*-mediated transformation. Directly transformed *B. rapa* plants carrying no *B. napus* crop-specific genes that would subsequently be introgressed in crop–weed hybrids. Thus, these plants are useful for testing transgene-specific effects in wild germplasm. Second, we characterized the transgenic events using molecular methods. Third, this study also began to test the effects of crop genetic load in introgressed crop–weed hybrids by comparing productivity of these transgenic *B. rapa* events to crop–weed hybrids under greenhouse and field conditions. Our goal is to better understand the roles of transgenesis, transgene expression, and gene flow on transgenic crop–weed fitness and persistence.

Materials and methods

A synopsis of *Brassica* plant types utilized in this study is provided in Table 1. Essentially we used an oilseed crop-type *B. napus* cv. 'Westar' with transgenic derivatives and weedy *B. rapa* accessions from Quebec, Canada and California, USA with transgenic derivatives, and backcrossed hybrids between the two species.

Plant transformation

Plant accessions

Plant material from three weedy accessions of *B. rapa* was used for plant transformation, including CA from Irvine, CA, USA (33°40′N 117°49′W): courtesy of Art Weis, QC2974 from Milby, QC, Canada (45°19′N 71°49′W), and QC2975 from Waterville, QC, Canada (45°16′N 71°54′W): courtesy of Suzanne Warwick.

Vectors

Two gene constructs were used for plant transformation, including pBin-mGFP5-ER (GFP gene only) and pSAM12 (*mGFP5-ER*-Bt *cry1Ac*; described in Harper et al. 1999). The pSAM12 construct contains *mGFP5-ER*, synthetic Bt *cry1Ac*, and kanamycin resistant *nptII* genes carried in the T-DNA, enabling all three traits to be inserted into a single, genetically linked locus. The Bt and GFP transgenes were expressed under the control of separate CaMV 35S promoter in separate cassettes on a single T-DNA vector.

Weedy B. rapa transformation

The transformation method and tissue culture system were based on existing protocol for *B. napus* transformation (Stewart et al. 1996). All cultures were maintained at 24 ± 2°C under a 16/8 h light/dark photoperiod. Rooted shoots were transferred to soil for an acclimation period of 2 weeks. Following acclimation, transgenic *B. rapa* plants were grown separately in 4 l pots filled with soil in a growth chamber. Number of explants, GFP sectors, shoots recovered, rooted shoots, and fertile plants were recorded to assess efficiency. Because of self-incompatibility of *B. rapa* plants, T_1 seeds were produced by hand-crossing between T_0 transgenic *B. rapa* and the respective accession wild *B. rapa* in growth chambers. T_1 plants were grown for 2 weeks and transgenic T_1 plants were retained if determined GFP positive by screening under a hand-held long wave ultraviolet (UV) light (UVP model B-100AP 100 W 365 nm). Their degree of green-fluorescence was indicative of moderate-to-high transgene expression. Hand-crossing among 36 individuals of the selected GFP positive T_1 plants produced T_2 seeds. A subsample of randomly chosen T_2 seeds was planted from each transgenic event, and after 2 weeks, seedlings were screened under UV light for positive GFP.

Southern blot analysis

Southern blot analysis was performed to confirm transgene integration in directly transformed T_2 *B. rapa* plants. Genomic DNA was extracted from frozen leaf tissue of GFP transgenic events and wild *B. rapa* using CTAB (Dellaporta et al. 1983). After digestion of 10 μg of genomic DNA with *Hin*dIII, fragments were purified with QIAquick PCR purification columns (QIAGEN, Valencia, CA, USA). Control plasmid DNA from the binary vector

Table 1 List of plant biotypes used in this study

Plant biotype	Construct	Event	Generation	Trans-genic	Name used in this study	Used in greenhouse study	Used in field study
B. napus (cv.Westar)			Parent		Westar		√
B. napus (cv.Westar)	pSAM12	Event1	T_4	√	BnGT1		√
BnGT1× *B.rapa* (QC2974)	pSAM12		BC_2F_2	√	Bt BC_2F_2	√	√
BnGT1× *B.rapa*(QC2974)			BC_2F_2		BC_2F_2	√	√
B. rapa (ac.QC2974)			Parent		QC2974	√	√
B. rapa (ac.QC2974)	pBIN-mGFP5-ER	Event2	T_2	√	74-GFP2		√
B. rapa (ac.QC2974)	pBIN-mGFP5-ER	Event3	T_2	√	74-GFP3	√	√
B. rapa (ac.QC2974)	pBIN-mGFP5-ER	Event5	T_2	√	74-GFP5		√
B. rapa (ac.QC2974)	pSAM12	Event1	T_2	√	74-GT1		√
B. rapa (ac.QC2974)	pSAM12	Event2	T_2	√	74-GT2	√	√
B. rapa (ac.CA)			Parent		CA		
B. rapa (ac.CA)	pBIN-mGFP5-ER	Event1	T_2	√	CA-GFP1		
B. rapa (ac.CA)	pSAM12	Event1	T_2	√	CA-GT1		
B. rapa (ac.QC2975)			Parent		QC2975		
B. rapa (ac.QC2975)	pSAM12	Event1	T_2	√	75-GT1		
B. rapa (ac.QC2975)	pSAM12	Event2	T_2	√	75-GT2		

Three weedy *B. rapa* accessions and one *B. napus* cultivar were used as parents. Two different constructs (pSAM12 contains GFP/Bt (GT) genes and pBIN-mGFP5-ER contains GFP gene) were used. Both transgenes of interest were under the control of the CaMV 35S promoter

pBIN-mGFP5-ER was also digested with *Hin*dIII. The *Hin*dIII was chosen as a restriction enzyme since *Hin*dIII cuts once within the pBIN-mGFP5-ER T-DNA, 5′ to the CaMV 35S promoter. DNA fragments were separated on a 1% agarose gel. Fragments were transferred to Zeta-Probe GT membrane (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions. A PCR product containing the full-length open reading frame of *mGFP5-ER* was radio-labeled with α-^{32}P dCTP using Prime-It II Random Primers Labeling Kit (Stratagene, La Jolla, CA, USA). Labeled probe was purified using mini Quick Spin DNA columns (Roche Diagnostics, Indianapolis, IN, USA). Southern blots were hybridized with labeled probe in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA) and washed according to manufacturer's protocol. Hybridized signal was visualized by exposure to phosphor-imaging screens (Storage Phosphor Screen GP, Eastman Kodak, Rochester, NY, USA) and scanned using Personal FX (Bio-Rad). Image analysis was undertaken using Quantity One software (Bio-Rad).

GFP fluorescence detection and analysis

Quantification of GFP fluorescence was performed using a Fluoromax -2 fluorescence spectrophotometer (Instruments S.A., Edison, NJ, USA) utilizing *DataMax* software (Galactic Industries Corporation, Salem, NH, USA). A 2 m bifurcated fiber cable was used to transmit excitation light and detect emission transmission from the leaves (Millwood et al. 2003). Fluorescence spectrometry was performed in the middle of third leaf at 4-leaf stage of GFP *B. rapa* events (transformed *B. rapa* with GFP gene), GT *B. rapa* events (transformed *B. rapa* with GFP/Bt genes), Bt BC_2F_2 hybrid, and wild *B. rapa* plants. Eight T_2 plants from each independent transgenic event were excited with UV light (385 nm) and scanned from 440 to 600 nm. For this study, the 450 nm wavelength served as the anchor point (Millwood et al. 2003). GFP fluorescence of the transgenic *B. rapa* events at 508 nm was subtracted from the anchor point average value for multiple measurement of wild *B. rapa* plants.

Bt expression analysis

Expression of Bt Cry1Ac protein in the transgenic *B. rapa* events was quantified by Bt enzyme-linked immunosorbent assay (ELISA) using a Qualiplate™ kit for Cry1Ab/Cry1Ac (Envirologix Inc, Portland, ME, USA). Total soluble protein was extracted from leaves using the protein extraction method described in Stewart et al. (1996). Fresh leaf tissue (0.2 g) from the transgenic *B. rapa* events and Bt BC_2F_2 (transgenic backcrossed generation between GT transgenic *B. napus* and *B. rapa* QC2974; described in Halfhill et al. 2005) was collected and homogenized in 400 µl of 0.1 N NaOH using a power drill in a microcentrifuge tube. The samples were incubated on ice for 30 min, and 80 µl of 1 M Tris–HCl (pH 4.5) was then added for neutralization. Each sample was clarified by

centrifugation at 8,765*g* for 5 min. The supernatant was discarded and the remaining fraction was quantified by Coomassie Plus™ Bradford Assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Soluble protein from each sample was diluted to 50 μg of protein per ml and 5 μg of soluble protein was put into each respective sample well. Quantification of Bt protein by ELISA was then performed using a Qualiplate™ kit for Cry1Ab/Cry1Ac according to manufacturer's instructions.

Transgenic *B. rapa* plants under insect pressure in greenhouse

To compare the productivity of transgenic *B. rapa* with crop–weed hybrids under herbivory pressure, a greenhouse experiment was performed at the Racheff research greenhouse at the University of Tennessee at Knoxville in the spring of 2005. Because *B. rapa* is self-incompatible, honeybees (*Apis mellifera*), were used as pollinators during the experiment.

Plant types

Five plant types used in this study; wild *B. rapa* (QC2974), transgenic GFP *B. rapa* event (74-GFP3), transgenic GT *B. rapa* event (74-GT2), transgenic backcrossed segregants between transgenic *B. napus* containing GFP and Bt transgenes (BnGT1) and QC2974 (Bt BC_2F_2), and non-transgenic backcrossed segregant (BC_2F_2; described in Halfhill et al. 2005; Table 1) were planted. One single plant was planted in each 4 l pot, and 30 pots were designated to each plant biotype. After 14 days, three different transgenic plants including 74-GFP3, 74-GT2, and Bt BC_2F_2 were screened under UV light in the dark to select for transgenics. GFP positive seedlings were retained and grown to maturity.

Plot design and application of insects

One hundred and fifty 4 l pots were arranged based on a randomized complete block design (RCBD) with sampling. Fifty pots were assigned to each block. Ten pots were designated to each plant type and each plant type was divided into two groups which included five pots per group. One of these groups was randomly placed either under herbivory pressure or not. A Bt susceptible strain diamondback moth (DBM) (*Plutella xylostella*) (Benzon Research Inc. Carlisle, PA, USA) was used as the herbivore. At the 8–12 leaf stage, 10 neonate DBM at the 3rd instar were applied to each plant using a small paint brush. After 2 weeks, the insecticide Marathon® 1% Granular (Olympic horticultural products Co., Mainland, PA, USA) highly toxic to DBM was applied to all plants in order to prevent immigration of DBM to non-treated plants, and to prevent further damage to treated plants.

Quantifying herbivory damage

Seven days after DBM application, the number of damaged leaves out of total countable leaves greater than 3 cm diameter was recorded. The percentage of damaged area on each leaf was estimated by visual assay based on the following categorical scale of damage (1 = no damage; 2 = <1% damage; 3 = <5% damage with 1 attempt; 4 = <5% damage with more than 1 attempt; 5 = 6–20% damage; 6 = 21–50% damage; 7 = 51–90% damage; 8 = >90% damage) (Halfhill et al. 2005).

Harvesting and analyses

At plant maturity, above-ground biomass was harvested and stored separately in mesh bag for a month at 36 ± 2°C until completely dried. Most plants were cured from powdery mildew infection by treating with Milstop fungicide (Bioworks Inc., Fairport, NY, USA), but a few plants that were still infected or dead were excluded from this analysis. Three plants were randomly selected from each five plants group for sampling. Dry weight of each selected individual plant was recorded and the number of total seeds per plant was calculated based on average weight of 100 seeds. Vegetative and reproductive productivity were estimated by plant dry weight and number of seeds, respectively. Plant productivity data among plant types and for insect application and no insect application treatments were analyzed by analysis of variance (ANOVA) using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA).

Productivity of transgenic *B. rapa* events under field conditions

To compare the productivity between transgenic *B. rapa* events and crop–weed hybrids under field conditions, a field experiment was performed in the spring of 2005 at the Lang Research Farm, Tifton, GA, USA (31°27′N 83°30′W).

Plant types

Ten plant types were used in this study; wild *B. rapa* (QC2974), three events of transgenic GFP *B. rapa* (74-GFP2, 74-GFP3, and 74-GFP5), two events of transgenic GT *B. rapa* (74-GT1 and 74-GT2), Bt BC_2F_2, and BC_2F_2 crop–weed hybrids, homozygous transgenic *B. napus* for GFP/Bt genes (BnGT1; described in Halfhill et al. 2001), and *B. napus* (cv. Westar; Table 1). All transgenic *B. rapa* events and Bt BC_2F_2 hybrids were screened with a hand-

held UV light in the dark to confirm the GFP transgene expression.

Plot design

The field experiment was designed based on an RCBD with eight replicates. A total of 80 plots were sown (10 plant types × 8 replicates). Each plot size was 1 m^2 and the isolation between plots was 1 m. Seeds were scattered by hand into each respective plot at a density of 200 seeds per m^2, then the seeds were raked in. At 4–8 leaf stage of plants, all GFP and GT events of transgenic *B. rapa* plants and Bt BC_2F_2 were screened with a hand-held UV light. The plant number in each plot was thinned randomly to 15 plants. Around our field experimental plots, border plants were planted at the margin of the plots to minimize the marginal effect.

Harvesting and statistical analyses

At plant maturity, above-ground vegetative biomass from each plot was harvested separately. Plant productivity was measured and data was analyzed as described previously in greenhouse experiment. Differences of productivity among plant types were compared by ANOVA and Contrast using SAS version 9.1 (SAS Institute Inc, Cary, NC, USA).

Results

Weedy *B. rapa* transformation and regeneration

Fifteen independent transgenic *B. rapa* events were generated from three weedy *B. rapa* accessions (QC2974, QC2975 and CA). The pBIN-mGFP5-ER and pSAM12 constructs generated 21 and 14 independent fluorescent sectors, respectively (Table 2). Roots formed on shoots from all accessions with both constructs. Wild *B. rapa* QC2974 transformed with pBIN-mGFP5-ER construct had relatively higher transformation efficiency than other accessions based on fertile plants from total explants. Transformed plants were confirmed by GFP screening under UV light, and were distinguished from wild *B. rapa* plants. Each T_1 transgenic *B. rapa* event segregated for GFP expression, except one 74-GFP event in which all progeny expressed GFP indicating multiple T-DNA insertions (data not shown—this event was not used in further characterizations). Only 9 transgenic *B. rapa* events out of 15 total established events were used for characterization because of the limited availability of T_2 seeds. The characterized events, based on GFP fluorescence, were all deemed as high expressing for transgenes.

Number of transgenes integrated in transgenic *B. rapa*

Independent T_2 GFP transgenic *B. rapa* events were analyzed by Southern blot analysis using *Hin*dIII digested genomic DNA (Fig. 1). Since *Hin*dIII restricts the T-DNA insert 5′ to the *mGFP5-ER* gene, probing with GFP yields an expected single hybridizing band for each T-DNA insert. Southern blot analysis results confirmed multiple transgene integrations in several events, including 74-GFP2, with two insertions, and 74-GFP5 and CA-GFP1, which both appear to contain four T-DNA inserts. The hybridizing bands in genomic digests varied in size between transgenic events and were not identical to hybridizing bands in *Hin*dIII digested binary vector control, indicating the different GFP transformed plants represented independent events and that transgenes were stably integrated in the *B. rapa* genome.

GFP fluorescence of transgenic *B. rapa* events

GFP expressions from transgenic *B. rapa* T_2 plants of each GFP and GT events were measured using fluorescence spectrometry. There were significant differences for emission intensity at 508 nm among transgenic *B. rapa* events including Bt BC_2F_2 (ANOVA, $P < 0.05$; Fig. 2). Transgenic *B. rapa* plants and Bt BC_2F_2 had a GFP expression at 508 nm from 2.5 ± 0.4 to 3. ± 6±0.3 cps (10^5) (all units in 10^5 counts per second). CA-GFP1 event exhibited the highest average 508 nm emission peaks at 3.6 ± 0.3 cps (10^5) (Fig. 2).

Bt Cry1Ac protein quantification

Transgenic GT *B. rapa* events and Bt BC_2F_2 hybrid expressed Bt Cry1Ac protein at varying levels from 0.016 to 0.045% of total soluble protein (Fig. 3). As expected, no Bt protein was detected from wild *B. rapa* and transgenic GFP *B. rapa* events. Significantly different Bt Cry1Ac concentrations were shown among independent GT *B. rapa* events (ANOVA, $P < 0.05$). Bt BC_2F_2 hybrid expressed less Bt Cry1Ac protein than transgenic GT *B. rapa* events (ANOVA, $P < 0.05$).

Productivity of transgenic *B. rapa* events under insect pressure

Estimated herbivory damage on leaves by visual assay varied between plant types. Little herbivory damage was observed on plants containing the Bt transgene, including 74-GT2 and Bt BC_2F_2 plants; however, non-Bt plants, QC2974, 74-GFP3, and BC_2F_2, had significant herbivory damage that was between 4 and 5 of categorical scale of

Table 2 Summary of weedy *Brassica rapa* accessions transformation efficiency using *Agrobacterium*-mediated method

Accession	Construct	Explants[a]	Sectors[b]	Shoots[c]	Roots[d]	Fertile[e]
QC2974	pBIN-mGFP5-ER	451	71	19	7	5
	pSAM12	625	98	11	4	2
QC2975	pBIN-mGFP5-ER	347	53	9	3	2
	pSAM12	445	37	9	5	4
CA	pBIN-mGFP5-ER	204	43	3	1	1
	pSAM12	276	39	1	1	1

Three weedy accessions of *B. rapa* were transformed with two different constructs including pBIN-mGFP5-ER and pSAM12. The pBIN-mGFP5-ER contains GFP gene and the pSAM12 contains GFP/Bt genes; both transgenes of interest were under the control of the CaMV 35S promoter

[a] Number of chopped hypocotyl segments initially infected by *Agrobacterium* inoculum

[b] Number of GFP fluoresced sectors

[c] Number of shoots regenerated

[d] Number of shoots that formed roots

[e] Number of transgenic plants that produced T_1 seeds

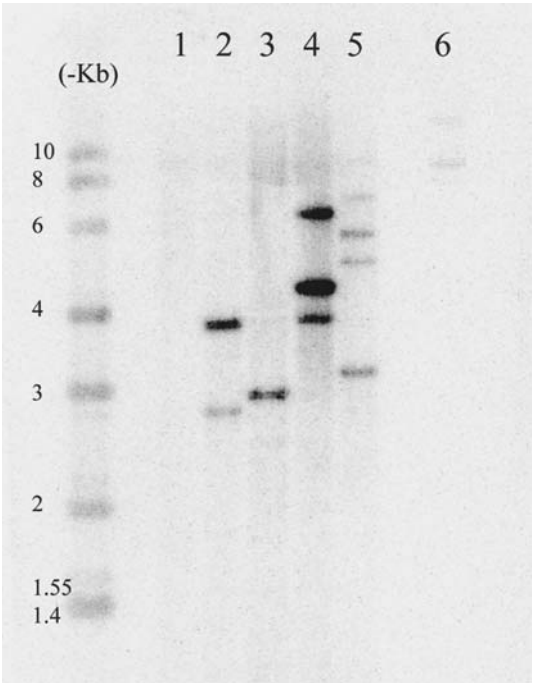

Fig. 1 Southern blot analysis of T_2 GFP *B. rapa* events. Southern blot analysis of *Hin*dIII digested genomic DNA hybridized to GFP probe. Genomic DNA from untransformed *B. rapa* QC2974 (lane *1*), three independent GFP transgenic events of *B. rapa* QC2974 (74-GFP2, 74-GFP3, 74-GFP5, lanes *2–4*), and a GFP transgenic event of *B. rapa* CA (CA-GFP1, lane *5*). *Hin*dIII digested binary vector pBIN-mGFP5-ER used for the transformation of *B. rapa* is shown in lane *6*

damage (ANOVA, $P < 0.05$). Herbivory damage was observed only in insect treated plants.

When the comparison of vegetative productivity was made within a single biotype plant, most biotypes had similar vegetative weight in the presence or absence of insect pressure (Fig. 4). Significant difference was detected for vegetative productivity among different plant types (ANOVA, $P < 0.05$). Bt BC_2F_2 had an unexpected low vegetative productivity under herbivory pressure. This result might be influenced by powdery mildew at plant maturity that especially affected the insect treated Bt BC_2F_2 plants. As an aside, backcrossed transgenic plants were found to not be as robust as their parental lines in a previous study (Halfhill et al. 2005), which might have made them more susceptible to powdery mildew. Wild *B. rapa* and BC_2F_2 plants without the Bt transgene grown under herbivory pressure had a lower reproductive productivity (ANOVA, $P < 0.05$) than those grown under non-insect treatment. There were no significant differences for reproductive productivity in the 74-GT2 and Bt BC_2F_2 events with and without herbivores (Fig. 4). The Shapiro–Wilk value from the ANOVA was greater than 0.95 indicating data need not be log-transformed for analysis.

Transgenic *B. rapa* plants productivity under field conditions

In contrast with the greenhouse experiment, no herbivores were added in the field and very little herbivore damage was observed on any *Brassica* plants based on visual survey during the experiment period. Significant differences in vegetative productivity between different plant types under field conditions were observed (ANOVA, $P < 0.05$; Fig. 5). No significant differences in vegetative productivity between wild QC2974 and transgenic *B. rapa* events were observed (Contrast, $P = 0.53$). Both Bt BC_2F_2 and BC_2F_2 crop-weed hybrids were not significantly different from wild QC2974 for vegetative productivity (Contrast, $P = 0.2$). No significant difference in reproductive pro-

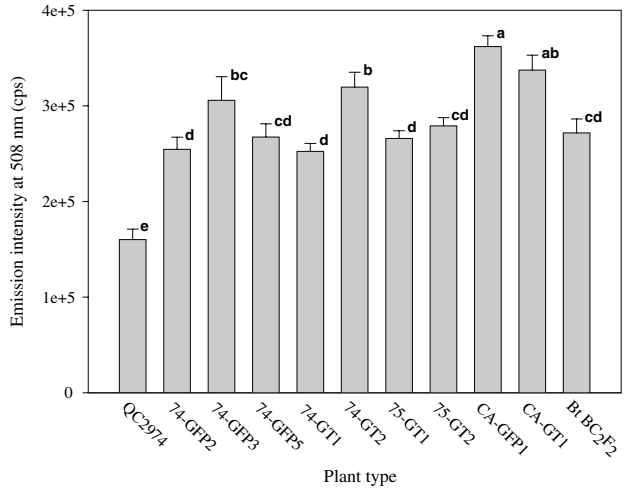

Fig. 2 Fluorescence average at 508 nm for T_2 independent transgenic *B. rapa* events and Bt BC_2F_2 hybrid. Wild weedy accession (QC2974) *B. rapa* served as a experimental control. Emission intensity was recorded in counts per second (cps) (10^5). *Different letters* represent significant difference between different plant types (ANOVA, $P < 0.05$). Error bars represent ± standard error of the means

ductivity was detected between different plant biotypes (ANOVA, $P = 0.65$; Fig. 5). Westar and BnGT1 produced similar number of seeds per plot to all *B. rapa* plants and crop–weed hybrids; however, both had significantly higher vegetative dry weight than all *B. rapa* plants and crop–weed hybrids (ANOVA, $P < 0.05$).

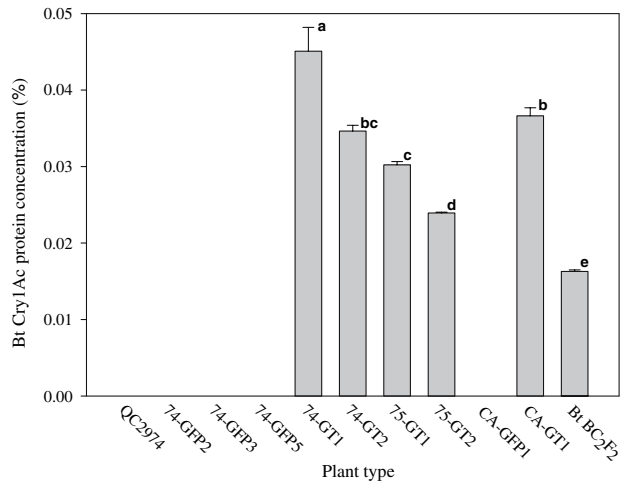

Fig. 3 Bt Cry1Ac protein concentration of transgenic *B. rapa* events and Bt BC_2F_2 hybrid from enzyme-linked immunosorbent assay (ELISA). *Different letters* represent significant differences between independent transgenic events (ANOVA, $P < 0.05$). Error bars represent ± standard error of the means

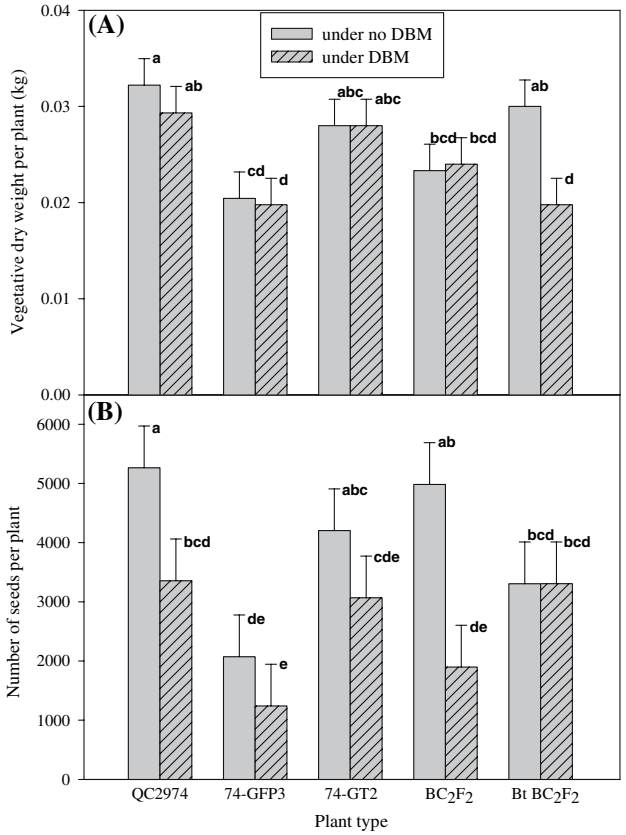

Fig. 4 Productivity of transgenic *B. rapa* events in greenhouse with and without diamondback moth (DBM) pressure. Five plant types (wild QC2974, 74-GFP3, 74-GT2, BC_2F_2, and Bt BC_2F_2 hybrids) were placed into groups of five pots per plant type and treatment in the greenhouse. Three pots were randomly sampled out of grouped five pots for analysis. **a** Represents vegetative productivity, and **b** represents reproductive productivity. *Different letters* represent significant differences among different plant types and DBM treatments (ANOVA, $P < 0.05$). Error bars represent ± standard error of the means

Discussion

Transformation of weedy *B. rapa* accessions

Transgenic *B. rapa* were produced to control for the absence of crop (*B. napus*) genes in subsequent assessments of backcrossed transgenic *B. napus* into the *B. rapa* genetic background. That is, the directly transformed transgenic *B. rapa* plants will contain the transgenes but no *B. napus* 'contaminating' genes, which are present in *B. napus*-transgenic backcrosses to *B. rapa* (Halfhill et al. 2003a, 2005). The BC_2 generation has a stable chromosome number of 20, as does *B. rapa* (Halfhill et al. 2005). Directly transformed *B. rapa* plants should be useful in biotechnology risk assessment research.

Brassica rapa is known as one of the most recalcitrant members of *Brassica* genus to regenerate shoots in vitro

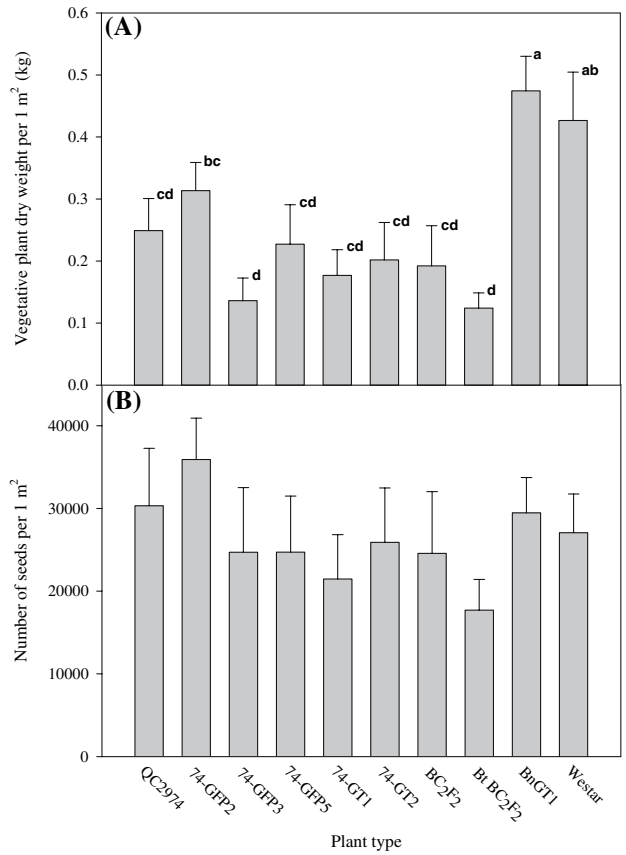

Fig. 5 Vegetative and reproductive productivity of transgenic *B. rapa* events under field conditions. Wild QC2974, 74-GT and GFP events, BC_2F_2, Bt BC_2F_2 hybrids, and *Brassica napus* (BnGT1 and non-transgenic *B. napus*) were planted and grown under field conditions with little-to-no herbivorous insect pressure. **a** Represents vegetative productivity, and **b** represents reproductive productivity. *Different letters* in **a** represent significant differences between different plant types (ANOVA, $P < 0.05$). No significant difference in reproductive productivity was detected between different plant types (ANOVA, $P = 0.65$). Error bars represent ± standard error of the means

(Murata and Orton 1987; Narashimhulu and Chopra 1988). Nonetheless, several *B. rapa* crop types, such as *oleifera*, *chinensis*, *pekinensis* have been transformed via *Agrobacterium*-mediated methods (Kuvshinov et al. 1999; Wahlroos et al. 2003; Qing et al. 2000; Zhang et al. 1998). To our knowledge, this paper describes the first transformed weedy accessions of *B. rapa* to have been produced and characterized. Relatively low transformation efficiency of *B. rapa* was reported here (i.e., ca. 0.4% efficiency—less than one-tenth the rates compared with our typical transformation efficiency *B. napus*—e.g., see Halfhill et al. 2001; Cardoza and Stewart 2003). Transformation efficiency may have been influenced by several factors, including genotype, explant type, donor plant age, and the *Agrobacterium* culture parameters (Poulsen 1996). Kuvshinov et al. (1999) showed that the shoot recovery efficiency of *B. rapa* spp. *oleifera* was highly dependant on the tissue used as explants. Another possibility is that wild weedy *B. rapa* may be recalcitrant to transformation in vitro because of its weedy genetic background; further, the tissue culture and transformation conditions have never been optimized for weedy genotypes.

Characterization of transgenic *B. rapa* events

Relatively high GFP expressing T_2 plants were selected by visual assay for characterizations. These selected T_2 plants were assumed to be homozygous plants for GFP gene, since homozygous and hemizygous plants could be differentiated by visualization of different GFP intensity in *Nicotiana tabacum* (Molinier et al. 2000) and *B. napus* seedlings (Halfhill et al. 2003a). Stable inheritance of GFP and Bt transgenes in T_2 generations of transgenic *B. rapa* events was confirmed by GFP expression measurement and Bt Cry1Ac ELISA analysis. Many possible factors can account for different intensity of GFP fluorescence, including positional effect of inserted transgene, leaf age, or unknown physiological variability (Molinier et al. 2000; Halfhill et al. 2001, 2003b). Fluorescence intensity at 508 nm varied between the independent transformation *B. napus* events (Halfhill et al. 2003b), which are consistent with our results.

Zhu et al. (2004) reported that Bt protein content ranged 0.8–0.16 μg/g leaf tissue at the 3–5 leaf stage of transgenic OSR. However, Bt protein content in transgenic *B. rapa* and crop–weed hybrid plant ranged 0.179–0.660 μg/g leaf tissue at the 4-leaf stage. This difference is likely the result of weedy genetic background, positional effects, and number of transgenes inserted per event. The level of Bt protein out of total soluble protein (0.016–0.045%) in the transgenic plants was still sufficient to cause the mortality of DBM in the greenhouse. Although previous studies in transgenic GT *B. napus* events strongly associated GFP fluorescence intensity with Bt concentration at maturity (Halfhill et al. 2003b), in this study no correlation was detected between the GFP fluorescence and Bt concentration in transgenic GT *B. rapa* events. This discrepancy with previously described transgenic events could be a matter of sampling at different plant ages, as all our transgenic plants were analyzed 24 days after planting. No correlation between copy number of transgenes and GFP fluorescence intensity was observed which contrasts with Stewart et al. (1996) for Bt-transgenic *B. napus*. Another study by Hobbs et al. (1993) has reported that copy number of GUS transgene in tobacco was associated with transgene expression.

Toxicity of Bt transgene to diamondback moth (DBM) in weedy B. rapa

Insecticidal genes such as Bt *cry1Ac* have been used to genetically engineer many agricultural crops (Schuler et al. 1998). Several Bt crystal protein endotoxins have been proven effective in reducing insect damage in crop plants (Hofte and Whiteley 1989). Bt susceptible DBM strain was used in this study; however, several Bt *cry1Ac* resistant diamondback moth strains have been developed (Roush 1994; Metz et al. 1995; Tabashnik et al. 1993). Bt transgenic OSR might be used to help manage DBM infestations (Ramachandran et al. 1998). However, expression of Bt transgene in weed populations may increase the difficulty of natural weed control system under insect selection pressure. Transgene escape from crop species into wild weedy populations has been observed; the fitness or productivity of crop–weed hybrid populations caused by transgene escape has been studied (Hauser et al. 1998; Gueritaine et al. 2002; Warwick et al. 2003; Mason et al. 2003; Halfhill et al. 2005). In this study, expression of the Bt *cry1Ac* gene in Bt transgenic weedy plants and crop–weed hybrids was effective in limiting damage caused by DBM. *B. rapa*, crop–weed hybrids, and OSR; all have similar susceptibility to DBM (Halfhill et al. 2002, 2005; Mason et al. 2003; Zhu et al. 2004). In the prior study (Halfhill et al. 2005), it was noted that one missing plant type that would be useful for comparisons was a directly transformed *B. rapa*; the subject of this study and as discussed earlier.

Transgenic *B. rapa* productivity under greenhouse and field conditions

We performed initial comparisons in the greenhouse and field with various *Brassica* types with the same transgene constructs. Seed production from Bt transgenic OSR in DBM infested field plots was 15 times higher than the non-Bt OSR (Ramachandran et al. 1998). In this greenhouse study, seed production in Bt transgenic plants was only 2 times higher than that of the non-transgenic plants, but the herbivory pressure was less in the present greenhouse study. Halfhill et al. (2005) has reported that OSR had more insect damage on leaves than wild *B. rapa* under ambient herbivore conditions, but no differences among plant biotypes for herbivory damage was detected because few herbivorous insects were observed during the experiment in our field trial. Crop–weed hybrids, with or without insecticidal transgene, had a similar productivity with directly transformed *B. rapa* and wild *B. rapa* under the absence of herbivorous insect field conditions. Vacher et al. (2004) and Halfhill et al. (2005), however, have reported crop–weed hybrids containing the Bt transgene produced significantly fewer seeds than their non-transgenic parents in the absence of herbivore pressure.

Directly transformed weedy *B. rapa* can serve as negative experimental controls to assess linkage effects of *B. napus* genes in introgression experiments because the productivity of the Bt transgenic *B. rapa* events was not different from the parent *B. rapa* in the absence of herbivory. In the case of directly transformed weedy *B.rapa*, the transgene of interest lies in the midst of endogenous weedy genes, which contrasts with the case of introgressed transgenic weeds, which will contain crop genes linked to the transgenic locus (Halfhill et al. 2003a; Stewart et al. 2003).

Weedy transgenic insect resistant *B. rapa* has been produced and characterized molecularly and using greenhouse and field experiments, one of the first weedy species to be transformed. When herbivores were added, transgenic plants were protected from defoliation. There was no apparent fitness disadvantage to the transgenic plants; thus these plants should be appropriate tools in additional biotechnology risk assessment experiments.

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