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Additive transgene expression and genetic introgression in multiple green-fluorescent protein transgenic crop × weed hybrid generations

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Abstract The level of transgene expression in crop × weed hybrids and the degree to which crop-specific genes are integrated into hybrid populations are important factors in assessing the potential ecological and agricultural risks of gene flow associated with genetic engineering. The average transgene zygosity and genetic structure of transgenic hybrid populations change with the progression of generations, and the green fluorescent protein (GFP) transgene is an ideal marker to quantify transgene expression in advancing populations. The homozygous T_1 single-locus insert GFP/*Bacillus thuringiensis* (Bt) transgenic canola (*Brassica napus*, cv Westar) with two copies of the transgene fluoresced twice as much as hemizygous individuals with only one copy of the transgene. These data indicate that the expression of the GFP gene was additive, and fluorescence could be used to determine zygosity status. Several hybrid generations (BC_1F_1, BC_2F_1) were produced by backcrossing various GFP/Bt transgenic canola (*B. napus*, cv Westar) and birdseed rape (*Brassica rapa*) hybrid generations onto *B. rapa*. Intercrossed generations (BC_2F_2 Bulk) were generated by crossing BC_2F_1 individuals in the presence of a pollinating insect (*Musca domestica* L.). The ploidy of plants in the BC_2F_2 Bulk hybrid generation was identical to the weedy parental species, *B. rapa*. AFLP analysis was used to quantify the degree of *B. napus* introgression into multiple backcross hybrid generations with *B. rapa*. The F_1 hybrid generations contained 95–97% of the *B. napus*-specific AFLP markers, and each successive backcross generation demonstrated a reduction of markers resulting in the 15–29% presence in the BC_2F_2 Bulk population. Average fluorescence of each successive hybrid generation was analyzed, and homozygous canola lines and hybrid populations that contained individuals homozygous for GFP (BC_2F_2 Bulk) demonstrated significantly higher fluorescence than hemizygous hybrid generations (F_1, BC_1F_1 and BC_2F_1). These data demonstrate that the formation of homozygous individuals within hybrid populations increases the average level of transgene expression as generations progress. This phenomenon must be considered in the development of risk-management strategies.

Keywords Transgenic canola · *Brassica rapa* · Green fluorescent protein · AFLPs · Gene flow

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Introduction

Gene flow from transgenic canola (*Brassica napus* L., AACC, 2n=38) to different canola varieties and weedy relatives, is a potential and realistic risk of the implementation of biotechnology on an agricultural scale. Hybridization between transgenic canola varieties and wild relatives represents a path for transgenic phenotypes to be acquired by natural populations (Raybould and Gray 1993; Warwick et al. 1999). Canola cultivation results in significant volunteer populations during subsequent years, and transgenic volunteer populations, particularly herbicide-resistant volunteers, present additional management concerns (Légère et al. 2001; Simard et al. 2002). Several studies have demonstrated hybridization between canola and wild relatives in both close (*Brassica rapa* L., AA, 2n=20) and distant (*Raphanus raphanistrum* L., RrRr, 2n=18) crosses under agricultural conditions (Jorgensen and Anderson 1994; Scott and Wilkinson 1998; Chèvre et

al. 2000; Rieger et al. 2001; Halfhill et al. 2002; Warwick et al. 2003). Hybridization experiments involving transgenics have shown that transgenes will be expressed in hybrid plants, and that transgenic phenotypes should be expected in agricultural fields where crops may cross-pollinate with weedy species (Metz et al. 1997; Halfhill et al. 2001, 2002).

Previous reports of transgene expression in hybrid populations have focused on qualitative assessments of transgenic phenotypes, such as herbicide tolerance, insect resistance or marker genes (Mikkelsen et al. 1996; Metz et al. 1997; Harper et al. 1999; Chèvre et al. 2000; Halfhill et al. 2001). As more is understood about the importance of transgene copy number and population structure, quantitative assessments at the population level will become necessary to effectively evaluate potential risk. There have been mixed results when investigating the relationship between transgene zygosity and expression. Several studies have demonstrated additive transgene expression between homozygous and hemizygous progeny from an independent transformation event (Hobbs et al. 1990; Stewart et al. 1996; Niwa et al. 1999; James et al. 2002), while other studies demonstrate no differences in expression based on zygosity (Hobbs et al. 1990; Caligari et al. 1993; Scott et al. 1998; James et al. 2002). Interspecific hybridization initially generates hemizygous F_1 individuals with one copy of the transgene locus. Over time, the average zygosity of a hybrid population equilibrates as backcrossing and intermating occurs, resulting, in the absence of selection, in Hardy-Weinberg equilibrium. If differential transgene expression occurs in mixed populations composed of hemizygous and homozygous individuals, the average transgenic phenotype for the population may change in subsequent generations.

The canola × *B. rapa* model system has been used to demonstrate that a weedy, *B. rapa*-like phenotype can be recovered after several backcross generations. Previous studies have shown that the F_1 hybrid generation is triploid (AAC, 2n=29) and, after multiple generations of backcrossing, the ploidy level of these generations is reduced to the original diploid level of the weedy parent, *B. rapa* (Metz et al. 1997; Halfhill et al. 2002). Quantification of the genetic introgression of the canola genome into backcrossed generations has been reported using several DNA marker systems, such as RFLP (Jorgensen and Andersen 1994; Mikkelsen et al 1996), inter-SSR (Scott and Wilkinson 1998) and AFLP analysis (Hansen et al. 2001; Warwick et al. 2003). Hybridization may significantly change the genetic composition of weedy populations, and understanding the degree of crop gene introgression may help predict how introgressed populations will interact in agricultural and natural environments.

Assuming transgenes will persist in backcrossed populations, the next step in evaluating the biosafety of transgenic hybrid populations is to determine what factors control the levels of transgene expression and to quantify the degree of *B. napus* genetic introgression in hybrid generations. We report results that determine transgene expression within various hybrid generations using the green fluorescent protein (GFP), which has been shown to be a quantitative marker for studying transgene expression (Harper et al. 1999; Halfhill et al. 2001; Stewart 2001; Richards et al. 2003). GFP analysis allows for the estimation of transgene expression levels through non-destructive, in situ measurements with a fluorescence spectrophotometer. The results from these experiments will be used to describe how zygosity variation will correlate to transgene expression and protein synthesis within a hybrid population. In order to quantify genetic introgression, AFLP analysis was used to determine the degree of *B. napus* introgression into multiple backcross hybrid generations with *B. rapa*.

Materials and methods

Breeding nomenclature

Transgenic canola (*B. napus*) events were defined as the progeny of independently transformed plants recovered from tissue culture. The primary plant was designated T_0, and subsequent selfed generations followed the pattern T_1 (progeny of T_0), T_2 (progeny of T_1) and T_3 (progeny of T_2). Primary transgenic events transformed with a plasmid-containing GFP (*Aequorea victoria, mGFP5er* variant) and Bt (*Bacillus thuringiensis,* synthetic Bt *crylAc*) genes received the label "GT", and plants transformed with a plasmid-containing only GFP were labelled "GFP" (Haseloff et al. 1997; Halfhill et al. 2001). Nine transgenic GT (GT 1–9) lines and three GFP (GFP 1–3) were used in this study. Wild accessions of *B. rapa* were collected as naturally occurring populations, and the nomenclature followed the designation provided by the collector. In the crossing experiments, *B. rapa* accession × canola event produced the F_1 hybrid generation, and *B. rapa* × F_1 produced the BC_1F_1 hybrid generation. The self-incompatible *B. rapa* accessions were used as the pollen recipients.

Backcrossing

Twenty seven BC_1F_1 GFP/Bt hybrid generations (*B. rapa* Calif., 2974, 2975×GT 1-9) and nine GFP hybrid generations (*B. rapa* Calif., 2974, 2975×GFP 1-3) were hand-crossed with the respective parental accession of *B. rapa* (CA from Irvine, California, USA, courtesy of Art Weis; 2974 from Milby, Québec, Canada, and 2975 from Waterville, Québec, Canada, germplasm collection AAFC-ECORC, Ottawa) (Halfhill et al. 2001). The *B. rapa* accessions were used as the pollen recipients. Three transgenic plants from each BC_1F_1 generation were allowed to flower, and hand-crossing was performed by removing BC_1F_1 flowers and pollinating six *B. rapa* plants to generate a BC_2F_1 generation. The hand-crossing proceeded as long as all plants continued to flower. All seeds were collected from the *B. rapa* parents, and were germinated on moist filter paper and screened by visual, qualitative assay for GFP fluorescence using a hand-held, long-wave ultraviolet light (Spectroline high-intensity long-wave UV lamp, BIB-150P model, 350 nm).

Three BC_2F_1 generations (Calif.×GT1, 2974×GT1 and 2974×GT8) were used to generate BC_2F_2 Bulk generations by placing 27 individuals of each line in greenhouse enclosures with a pollinating insect, housefly (*Musca domestics* L.). Seeds of the BC_2F_1 generation were germinated in soil, and transgenic BC_2F_1 individuals were selected for GFP fluorescence by visual assay with a UV light. Plants from each line were isolated in greenhouse enclosures, and houseflies were added when plants began to flower. Due to the self-incompatibility of the BC_2F_1 plants, a pollinating

insect was required in order to generate a large number of random pollination events. Seeds were collected from all individuals within an enclosure and bulked together to form the BC_2F_2 Bulk generations.

Plant material for zygosity determination

Nine T_1 GFP/Bt events (GT 1–9), one GFP event (GFP 2), and non-transformed canola (*B. napus* cv Westar) described in Halfhill et al. (2001) were used to determine the effect of zygosity on GFP fluorescence. Twenty plants from each event were germinated on moist filter paper, and GFP fluorescence was detected by visual assay using a hand-held UV light. The number of fluorescent individuals was compared to non-fluorescent seedlings as an indicator of Mendelian segregation of the transgene. All plants were transplanted into soil in 4×4 inch pots and grown in controlled-environment growth chambers (Percival Scientific, Perry, Iowa, USA) at 22°/18°C under a 12/12 h light/dark cycle. All plants were uniformly fertilized on a weekly basis with a complete fertilizer. Quantitative fluorescence measurements were recorded at the 8-leaf stage from the youngest expanding leaf (fluorescence spectrophotometry described below).

Isolated plants were allowed to flower and were self-pollinated. Seeds were collected from each plant after reaching full maturity, and the zygosity of each parent was determined by progeny analysis of the T_2 seeds. Twenty seeds were germinated from each T_1 parent, and the progeny were screened for GFP fluorescence with a hand-held UV light. Plants were classified as homozygous if all T_2 seedlings were GFP fluorescent, and hemizygous if any non-fluorescent seedlings were recorded.

Fluorescence spectrophotometry

A Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon and Glen Spectra, Edison, N.J., USA) with DataMax and GRAMS/386 software (Galactic Industries Corporation, Salem, N.H., USA) was used to quantify GFP fluorescence of all plants in the study. Seeds from T_3 canola (GT1 and GT8), non-transformed canola (Westar), *B. rapa* (Calif. and 2974), and successive hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk) from three crossing lines (Calif.×GT1, 2974×GT1, and 2974×GT8) were germinated on filter paper and selected for GFP fluorescence. Ten plants were analyzed for each generation, except for the BC_2F_2 Bulk, where 30 plants were analyzed. From all plant lines, GFP fluorescent individuals were randomly selected, transplanted to soil, and moved to identical controlled-environment growth chambers. The youngest elongating leaf at the 8-leaf stage was excited at 385 nm slightly off the midvein on the underside of the leaf, and emission spectra were recorded from 420 to 600 nm. Intensity was measured at 508 nm (fluorescence maximum for the *mGFP5er* gene) in counts per second (cps). The 450 nm wavelength, outside the GFP fluorescence spectrum, was set as a baseline for each sample (Millwood et al. 2003). All samples were standardized to the average 450-nm value of non-transgenic canola to control for baseline variation.

Ploidy determination

Flow cytometry was used to estimate the ploidy level of the three BC_2F_2 Bulk generations produced in this study. Ten plants were analyzed from each generation. Leaves were selected from the two youngest leaf positions on each plant, and 0.5 g were removed and chopped with a razor blade in 3 mls of buffer. The extract was strained through a 15-micron nylon filter, and isolated nuclei were stained with 150 μl/ml of propidium-iodine in the dark for 10 min. The isolation and propidium-iodide staining of nuclei was performed according to Galbraith et al. (1983). Flow cytometry was performed using a Becton Dickinson FACS Caliber flow cytometer using the side scatter monitor to analyze the data. Each test sample included isolated parental nuclei as an internal control. The histograms in Fig. 1 were generated by recording the relative fluorescence at intervals of every 10 channels through channel 610.

AFLP analysis

Plant material

Plant material from *B. napus* (cv Westar) and three accessions of *B. rapa* (Calif., 2974 and 2975) were used as parental controls. A total of nine lines representing F_1 hybrid and backcross generations: BC_1F_1, BC_2F_1, BC_2F_2 Bulk for three crossing lines *B. rapa* × GT *B. napus* (Calif.×GT1, 2974×GT1 and 2974×GT8) were analyzed. Six samples per line were grown, young leaves harvested and stored at −80°C. Approximately 100 mg of previously frozen leaf material of each sample was freeze-dried for 3 days in a punctured, capped tube.

AFLP amplification

Individual samples were ground in a Fast Prep FP120 (BIO 101) grinder and the total genomic DNA extracted using a modified 2 × CTAB procedure (Doyle and Doyle 1987). AFLPs were generated based on the protocol of Vos et al. (1995) with minor modification. For each sample, approximately 250 ng of DNA was digested with 1.25 U of *Eco*R1 and 1.25 U of *Mse*1 (Invitrogen) in a 5× reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM MgAc, 250 mM KAc) in a reaction volume of 12.5 μl at 37°C for 2.5 h, and the restriction enzymes were heat inactivated at 65°C for 10 min. The digested DNA samples were ligated to adaptors in a ligation solution containing 0.5 U of T4 DNA Ligase, 5× ligation buffer (Invitrogen), 0.4 mM of ATP, 1 pMol of *Eco*R1 adapter and 10 pMol of *Mse*1 adapter (Cortec) in a reaction volume of 25.0 μl at 22°C for 2 h (Table 1). The adapter-ligated DNA was diluted 10-fold with

Table 1 Nomenclature and sequences of adapters and primers used for amplified fragment length polymorphism (AFLP) analysis

Eco RI adapter		*5' CTCGTAGACTGCGTACC 3'* *3' CATCTGACGCATGGTTAA 5'*
Eco RI + 1 primer	E+A	*5' GACTGCGTACCAATTCA 3'*
Eco RI + 3 primer	E+AAC	*5' GACTGCGTACCAATTCAAC 3'*
	E+AAG	*5' GACTGCGTACCAATTCAAG 3'*
Mse I adapter		*5' GACGATGAGTCCTGAG 3'* *3' TACTCAGGACTCAT 5'*
Mse I + 1 primer	M+C	*5' GATGAGTCCTGAGTAAC 3'*
Mse I + 3 primer	M+CAC	*5' GATGAGTCCTGAGTAACAC 3'*
	M+CAG	*5' GATGAGTCCTGAGTAACAG 3'*
	M+CAA	*5' GATGAGTCCTGAGTAACAA 3'*
	M+CAT	*5' GATGAGTCCTGAGTAACAT 3'*
	M+CTA	*5' GATGAGTCCTGAGTAACTA 3'*

TE buffer and used as a template for pre-amplification. Pre-amplification was carried out with primers complimentary to the *Eco*RI and *Mse*I adapters, with one selective nucleotide at the 3′ end (E+A and M+C, Table 1). The pre-amplification reaction included 2.5 μl of the diluted adapter-ligated DNA, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.5 U of *Taq* (Roche), 10× PCR buffer (Invitrogen), 7.5 ng of the E+A and M+C primers in a reaction volume of 10.0 μl. Amplifications were performed in a Techne Genius thermocycler following the PCR parameters in Vos et al. (1995). The pre-amplified DNA was diluted 4-fold with TE buffer and used as a template for selective amplification with *Eco*RI and *Mse*I primers, each having three selective nucleotides at the 3′ end (Tables 1 and 2). The selective amplification reaction included 1.25 μl of diluted pre-amplification product, 0.2 mM of dNTPs, 1.5 mM of $MgCl_2$, 0.5 U of *Taq* (Roche), 10× PCR buffer (no $MgCl_2$) (Invitrogen), 7.5 ng of the *Mse*I selective primer, and 0.125 μl of *Eco*RI selective primer labelled with an infrared dye IRD-700 (LI-COR) in a reaction volume of 5.0 μl. The PCR parameters for selective amplification followed Vos et al. (1995). The amplified product, mixed with an equal volume (5 μl) of loading buffer/formamide dye, was denatured at 94°C for 3 min and held at 4°C. PCR products were separated in a 5% polyacrylamide gel on an automated sequencer (LI-COR) for 5 h. Infrared gel images were analyzed using a GeneIR (Scanalytics) program (LI-COR). *B. napus*-specific markers, i.e., those present within all parental *B. napus* individuals and absent in all *B. rapa* individuals, were selected. The presence/absence of each marker was scored for all hybrid and backcross individuals.

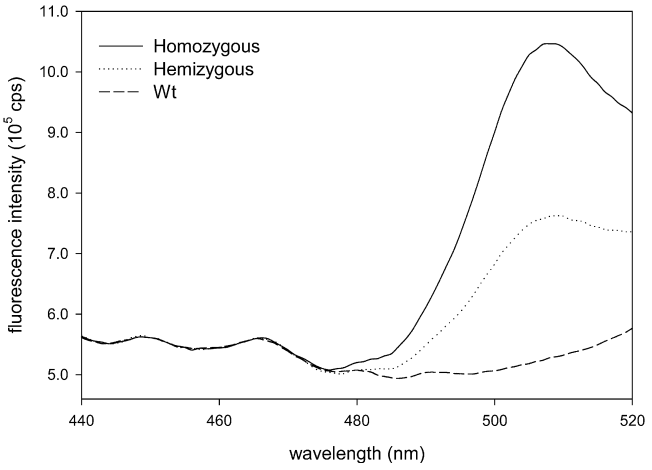

Fig. 1 Average scanning fluorescence-emission of homozygous versus hemizygous individuals of T_1 GFP2 canola when excited with 385-nm UV light. Non-transformed canola (Westar, *Wt*) was used as a control. Homozyogous individuals exhibited an average fluorescence at 508 nm of 10.5±0.4 (all units in 10^5 counts per s ± SD) compared to hemizygous individuals that had an average fluorescence of 7.6±1.4. Wt exhibited an average fluorescence of 5.3±0.7

Results

Zygosity determination and average whole-plant fluorescence

In each transformation event, the ratio of fluorescent to non-fluorescent T_1 seedlings was within the expected 3:1 ratio for a single-locus transgene insertion (chi-squared analysis, data not shown). GFP fluorescence of the T_1 plants within each transformation event was correlated to the zygosity of each individual (Figs. 1 and 2). Homozygous individuals demonstrated significantly higher fluorescence at 508 nm compared to hemizygous individuals (P<0.05). After standardization, the homozygous and hemizygous fluorescence profiles differed only at the magnitude of the GFP peak (480–540 nm) (Fig. 1). For example, GFP2 homozygous plants exhibited an average fluorescence of 10.5±0.4 (all units in 10^5 counts per seconds ± standard deviation) compared to hemizygous plants that had an average fluorescence of 7.6±1.4 cps (Fig. 1). Non-transformed canola exhibited an average fluorescence of 5.3±0.7. When the non-transgenic (Westar) level of fluorescence was removed from each sample, homozygous individuals fluoresced twice as much as hemizygous individuals above the background level of fluorescence. The difference between zygosity states was consistent and statistically significant within all ten transgenic lines analyzed in the study (Fig. 2).

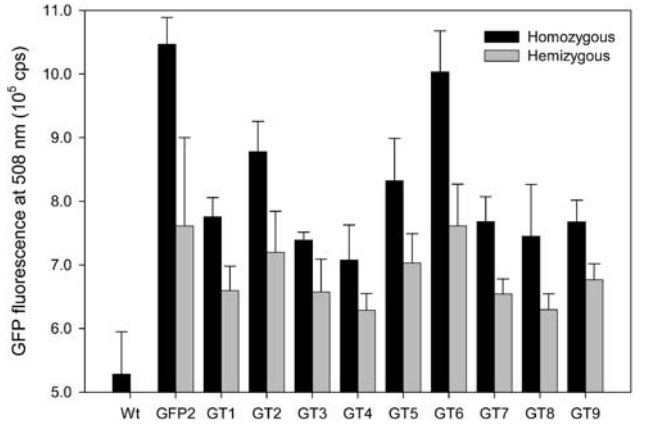

Fig. 2 GFP fluorescence at 508 nm for homozygous versus hemizygous T_1 plants within ten transgenic canola lines when excited with 385-nm UV light. Non-transformed canola (Westar, *Wt*) was used as a control. Homozygous individuals fluoresced significantly higher at 508 nm compared to hemizygous individuals (P<0.05)

Ploidy determination

The ploidy of the three BC_2F_2 Bulk generations was indistinguishable from *B. rapa* when analyzed through flow cytometry (ten plants per generation, 30 total) (Fig. 3). When parental canola and *B. rapa* nuclei were mixed, the respective G1 peaks were separated by about 250 relative fluorescence units (Fig. 3A). When mixed with canola, the BC_2F_2 Bulk generations exhibited similar G1 peak separation from canola as *B. rapa* (Fig. 3B). When mixed with *B. rapa*, resultant G1 and G2 peaks were identical and additive (Fig. 3C). These results indicate that when the ploidy is reduced from the F_1 hybrid level (29 chromosomes) to the *B. rapa* level (20 chromosomes) through two backcrosses (BC_1F_1 and BC_2F_1), the ploidy is stable after an intermating generation (BC_2F_2 Bulk).

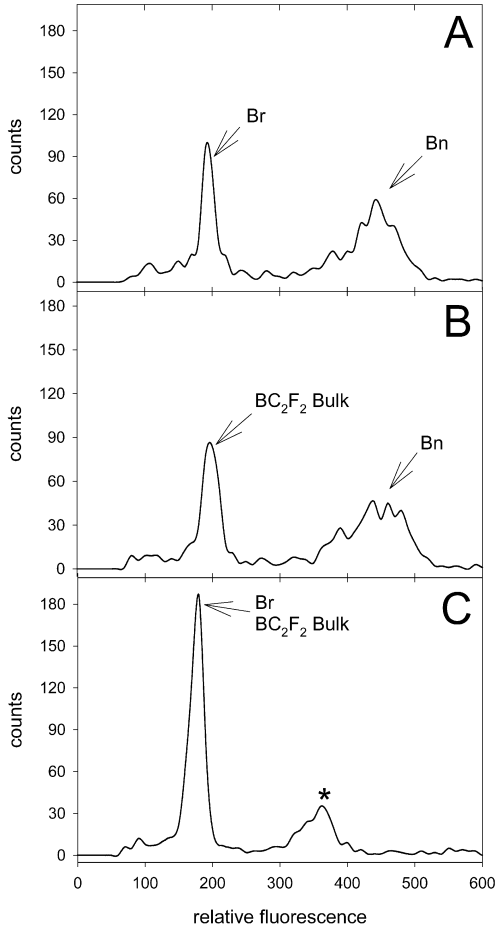

Fig. 3A–C Relative DNA content of canola (*B. napus*, *Bn*), birdseed rape (*B. rapa*, *Br*) and BC_2F_2 Bulk hybrids as determined by flow cytometry. Panel **A**, histogram of flow cytometric analysis of mixed samples of parental canola and birdseed rape nuclei. Panel **B**, histogram of BC_2F_2 Bulk nuclei mixed with canola, and panel **C**, histogram of BC_2F_2 Bulk nuclei mixed with birdseed rape. The *arrows* in each panel de-mark the respective G1 peak (2C) of each type, and the *asterisk* (*) represents the additive G2 peak in panel **C**. Note that G2 peaks for *Bn* are off the scale for panels **A** and **B**

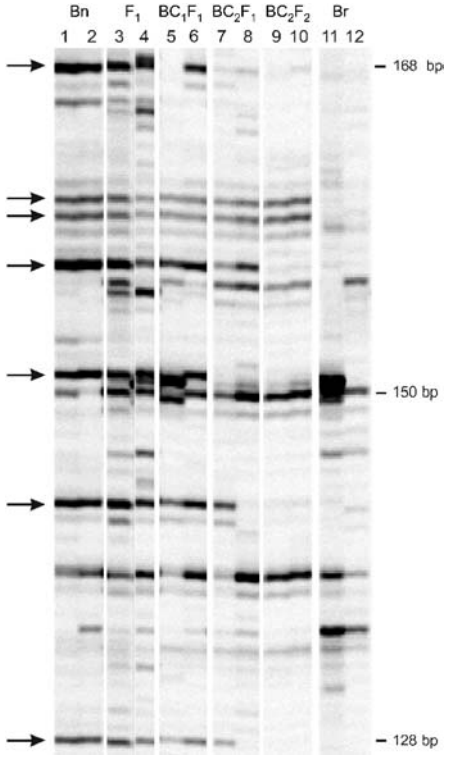

Fig. 4 AFLP analysis of canola (*B. napus,* GT1; Bn, *lanes 1–2*), hybrid generations (Calif.×GT1: F_1, *3–4*; BC_1F_1, *5–6*; BC_2F_1, *7–8*, BC_2F_2 Bulk, *9–10*), and *B. rapa* (Calif.; Br, *11–12*). *Arrows* represent *B. napus*-specific AFLP markers

Table 2 Primer combinations used for selective amplification and the total number of resolved AFLP markers

	Visible Bands	*B. napus*-specific markers
1. E+AAC/M+CAC	45	15
2. E+AAC/M+CAG	48	16
3. E+AAG/M+CAA	68	20
4. E+AAG/M+CAT	64	23
5. E+AAG/M+CTA	45	18
Totals	270	92

AFLP analysis

The five primer-pair combinations generated a total of 270 consistently amplified AFLP bands (Fig. 4), 92 of which were *B. napus*-specific markers (Table 2). Each hybrid generation was analyzed for the presence/absence of these *B. napus*-specific AFLP markers, and it was determined that the number of markers decreased with each backcross generation (Fig. 5). The F_1 generation contained between 95–97% of the *B. napus*-specific markers, and the BC_1F_1 generation contained a reduction of these markers to between 62%±12 and 75%±14 (percentage of *B. napus*-specific markers ± SD). The BC_2F_1 generation continued this general trend, but demonstrated a wide range of crop-specific markers between the crossing lines. The CA×GT1 and 2974×GT1 BC_2F_1 generations had lower percentages of these markers: 30%±7 and 25%±3, respectively, in contrast to the 2974×GT8 BC_2F_1 generation that had a high number of 73%±2 of the *B. napus*-specific markers. The BC_2F_2 Bulk populations sustained a reduction of *B. napus*-specific markers, and exhibited the lowest percentage of markers per crossing line ranging from 15%±1 to 29%±3 although the ploidy had not changed.

Average GFP fluorescence of multiple-backcross hybrid generations

All hybrid generations within each crossing line were analyzed for average GFP fluorescence (Fig. 6). In each crossing line, the homozygous canola plants exhibited significantly higher average GFP fluorescence when compared to the hemizygous hybrid generations (F_1,

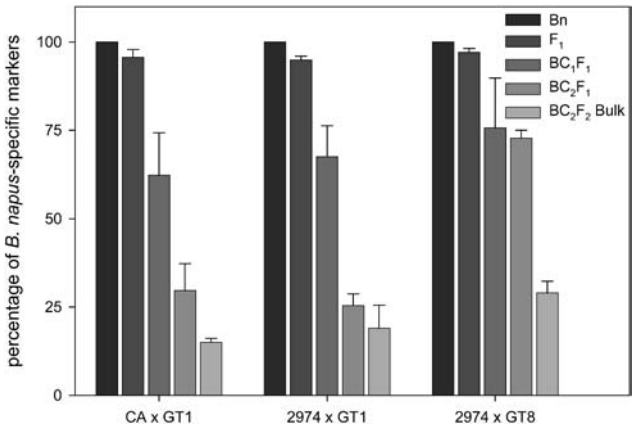

Fig. 5 AFLP analysis of canola (*B. napus*, *Bn*) and F_1, BC_1F_1, BC_2F_1 and BC_2F_2 Bulk hybrid generations. AFLP analysis with five specific primer sets yielded 92 *B. napus*-specific markers. Within each crossing line (Calif.×GT1, 2974×GT1, 2974×GT8), percentage of *Bn* specific markers±SD for each generation is shown

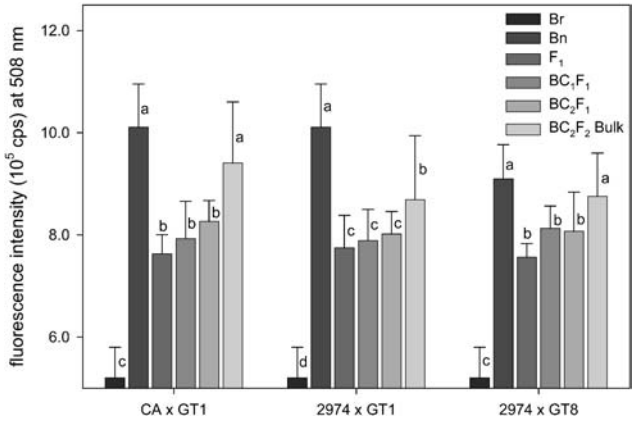

Fig. 6 Average GFP fluorescence of parental transgenic canola (*Bn*) and F_1, BC_1F_1, BC_2F_1 and BC_2F_2 Bulk hybrid generations with *B. rapa* (*Br*). Leaves were excited with 385-nm UV light and fluorescence intensity was measured at 508 nm (*cps*). Fluorescence averages (± standard deviation) per generation were compared by ANOVA within each crossing line (Calif.×GT1, 2974×GT1 and 2974×GT8), and *letters* indicate significant differences between generations (Fisher's PLSD, $P<0.05$)

BC_1F_1 and BC_2F_1) and the wild-type *B. rapa* parent (ANOVA, Fisher's PLSD, $P<0.05$). The average magnitude of fluorescence of GT1 was 10.1±0.8 (10^5 counts per second ± SD at 508 nm), and the hemizygous generations ranged in fluorescence from 7.6±0.3 to 8.2±0.5. The hemizygous hybrid lines were not significantly different from one another, and always exhibited significantly greater fluorescence than the *B. rapa* parent. The BC_2F_2 Bulk generation was composed of a mixture of homozygous and hemizygous individuals, and in all cases exhibited greater fluorescence than the hemizygous generations (Fig. 6). In crossing lines CA×GT1 and 2974×GT8, average GFP fluorescence of the BC_2F_2 Bulk generation was not significantly different from the original homozygous canola event.

Discussion

Additive transgene expression

The GFP gene demonstrated additive transgene expression in ten independent transformation events of canola. In all canola lines, homozygous individuals that contained two copies of the transgene locus fluoresced twice as much as hemizygous individuals above the background level of fluorescence. In previous studies (Hobbs et al. 1990; Caligari et al. 1993; Scott et al. 1998; Allen et al. 2000; James et al. 2002), independent transformation events modified with the same plasmid have been shown to exhibit a wide range of expression levels and stability through generations. Allen et al. (2000) and James et al. (2002) established that some transgenic events show transgene silencing while others are consistently expressed, and matrix attachment regions (MARs) were shown in each case to limit transgene silencing of a GUS transgene in tobacco and rice, respectively. In the canola lines analyzed in this study, no evidence of transgene silencing was found, and this may be due to the inclusion of a single tobacco RB7 MAR on the pSAM12 plasmid that was used for the GT canola transformations (Harper et al. 1999). The transgenic canola lines were also generated by positive selection for the GFP transgenic phenotype in tissue culture (Stewart et al. 2002). This type of selection could have possibly removed low-expressing transgenic events or those with the tendency to silence, and skewed the T_0 population towards events that were resistant to transgene silencing. The combination of positive selection and the inclusion of a MAR on the plasmid have produced a population of transgenic canola events that demonstrate additive transgene expression and resistance to transgene silencing.

Additive transgene expression of GFP has also been shown in two other plant models, tobacco (*Nicotiana tabacum* L.) and *Arabidopsis thaliana* (L.) Heynh. (Niwa et al. 1999; Molinier et al. 2000). In the tobacco model, classes based on fluorescence intensity (high, low and no fluorescence) were used to predict the zygosity status of segregating T_1 progeny. Our study could also categorize individuals based on a class level (data not shown), but the numerical quantification provided by fluorescence spectrophotometry allowed for a precise, quantifiable measure of the GFP phenotype. The precise measurement of the GFP phenotype could be useful in models used to estimate recombinant protein per unit area based on fluorescence (Richards et al. 2003). In these models, GFP fluorescence has been shown to correlate with the total amount of GFP present in water and plant extracts. In the construction of fusion proteins, in which GFP is fused to another recombinant protein, simple fluorescence measurements could predict the yield or phenotype of an otherwise immeasurable transgenic character. GFP fluorescence in transgenic *A. thaliana* lines has been quantified using blue laser-based spectrophotometry on a FluorImager imaging system (FluorImager SI, Molecular Dynamics). Although this system was also quantitative,

the instrument required small plant samples and therefore destructive sampling of larger material to fit within the instrument. With the Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon and Glen Spectra, Edison, N.J.), sampling was conducted on large plants with the use of a fiber-optic cable directly on intact plant tissues. Millwood et al. (2003) has recently reported the development of a portable fluorescence spectrophotometer (GFPMeter, OptiScience, Tyngsboro, Mass.) that is highly accurate and can be used under field conditions. This instrument allows for future applications of GFP to be utilized under large-scale agricultural conditions, and may expand the uses of GFP that have been limited by the requirement for lab-based quantitative systems.

GFP hybrid generations

The GFP transgenic phenotype was qualitatively useful in the selection of hybrid plants at the seedling stage, and allowed for the accurate selection of transgenic material in subsequent generations. Positive selection for the GFP phenotype is advantageous in comparative studies, because non-transgenic individuals can be germinated under identical conditions without the possibly confounding effects of tissue culture or destructive collection required for molecular-based confirmation analyses. In the future, GFP hybrid generations will allow for physiological analyses to determine fine-scale differences between populations, which may be used for risk assessment to predict the competitive ability of transgenic individuals.

In advanced hybrid generations, the average transgenic phenotype was shown to increase as intermating altered the degree of homozygosity within the population. The finding that later generations will reach the average transgenic phenotype of the crop variety must be included in risk-management strategies. After hybridization occurs and hemizygous F_1 individuals are produced, the population should be expected to shift the degree of the transgenic phenotype based on the dynamics of breeding within the hybrid population and weedy plants. Future research must quantify the frequency of backcrossing and selfing amongst transgenic individuals under field conditions to make predictions about the effects of the dynamic transgenic phenotype.

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References

Allen GC, Spiker S, Thompson WF (2000) Use of matrix attachment regions (MARs) to minimize transgene silencing. Plant Mol Biol 43:361–379

Caligari PDS, Yapabandara YMHB, Paul EM, Perret J, Roger P, Dunwell JM (1993) Field performance of derived generations of transgenic tobacco. Theor Appl Genet 86:875–879

Chèvre AM, Eber F, Darmency H, Fleury A, Picault H, Letanneur JC, Renard M (2000) Assessment of interspecific hybridization between transgenic oilseed rape and wild radish under agronomic conditions. Theor Appl Genet 100:1233–1239

Doyle JJ, Doyle JL (1987) A rapid DNA-isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Galbraith DW, Harkins KR, Maddox JM, Ayres NM, Sharma DP, Firoozabady E (1983) Rapid-flow cytometric analysis of the cell cycle in intact plant tissues. Science 220:1049–1051

Halfhill MD, Richards HA, Mabon SA, Stewart CN Jr (2001) Expression of GFP and Bt transgenes in *Brassica napus* and hybridization and introgression with *Brassica rapa*. Theor Appl Genet 103:362–368

Halfhill MD, Millwood RJ, Raymer PL, Stewart CN Jr (2002) Bt transgenic canola hybridization with its weedy relative, *Brassica rapa.* Environ Biosafety Res 1:19–28

Harper BK, Mabon SA, Leffel SM, Halfhill MD, Richards HA, Moyer KA, Stewart CN Jr (1999) Green-fluorescent protein as a marker for expression of a second gene in transgenic plants. Nature Biotech 17:1125–1129

Hansen LB, Siegismund HR, Jorgensen RB (2001) Introgression between oilseed rape (*Brassica napus* L.) and its weedy relative *B. rapa* L. in a natural population. Genet Res Crop Evol 48:621–627

Haseloff J, Siemering KR, Prasher DC, Hodge S (1997) Removal of a cryptic intron and subcellular localization of green-fluorescent protein are required to mark transgenic *Arabidopsis* plants brightly. Proc Natl Acad Sci USA 94:2122–2127

Hobbs SLA, Kpodar P, Delong CMO (1990) The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol Biol 15:851–864

James VA, Avart C, Worland B, Snape JW, Vain P (2002) The relationship between homozygous and hemizygous transgene expression levels over generations in populations of transgenic rice. Theor Appl Genet 104:553–561

Jorgensen RB, Andersen B (1994) Spontaneous hybridization between oilseed rape (*Brassica napus*) and weedy *B. campestris* (Brassicaceae): a risk of growing genetically modified oilseed rape. Am J Bot 81:1620–1626

Légère A, Simard M-J, Thomas AG, Pageau D, Lajeunesse J, Warwick SI, Derksen DA (2001) Presence and persistence of volunteer canola in Canadian cropping systems. Proc Brighton Crop Prot Conf—Weeds. British Crop Protection Council, Farnham, Surrey, UK, pp 143–148

Metz PLJ, Jacobsen E, Nap JP, Pereira A, Stiekema WJ (1997) The impact of biosafety of the phosphinothricin-tolerance transgene in inter-specific *B. rapa* × *B. napus* hybrids and their successive backcrosses. Theor Appl Genet 95:442–450

Mikkelsen TR, Andersen B, Jorgensen RB (1996) The risk of crop transgene spread. Nature 380:31

Millwood RJ, Halfhill MD, Harkins D, Russotti R, Stewart CN Jr (2003) Instrumentation and methodology of GFP quantification in intact plant organs. Biotechniques 34:638–643

Molinier J, Himber C, Hahne G (2000) Use of green-fluorescent protein for detection of transformed shoots and homozygous offspring. Plant Cell Rep 19:219–223

Niwa Y, Hirano T, Yoshimoto K, Shimizu M, Kobayashi H (1999) Non-invasive quantitative detection and applications of non-toxic, S65T-type green-fluorescent protein in living plants. Plant J 18:455–463

Raybould AF, Gray AJ (1993) Genetically modified crops and hybridization with wild relatives: a UK perspective. J Appl Ecol 30:199–219

Richards HA, Halfhill MD, Richards RJ, Stewart CN Jr (2003) GFP fluorescence as an indicator of recombinant protein expression in transgenic plants. Plant Cell Rep 2:150–158

Rieger MA, Potter TD, Preston C, Powles SB (2001) Hybridization between *Brassica napus* L. and *Raphanus raphanistrum* L. under agronomic field conditions. Theor Appl Genet 103:555–560

Scott A, Woodfield D, White DWR (1998) Allelic composition and genetic background effects on transgene expression and inheritance in white clover. Mol Breed 4:479–490

Scott SE, Wilkinson MJ (1998) Transgene risk is low. Nature 393:320

Simard MJ, Légère A, Pageau D, Lajeunnesse J, Warwick S (2002) The frequency and persistence of canola (*Brassica napus*) volunteers in Québec cropping systems. Weed Technol 16:433–439

Stewart CN Jr (2001) The utility of green-fluorescent protein in transgenic plants. Plant Cell Rep 20:376–382

Stewart CN Jr, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D, Parrott WA (1996) Genetic transformation, recovery, and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringiensis cryIAc* gene. Plant Physiol 112:121–129

Stewart CN Jr, Halfhill MD, Millwood RJ (2002) GFP in transgenic plants: *Brassica* transformation. In: Hicks B (ed) Green fluorescent protein, methods and protocols. Methods in Molecular Biology, Humana Press, Totowa, New Jersey, pp 245–252

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Warwick SI, Beckie H, Small E (1999) Transgenic crops: new weed problems for Canada? Phytoprotection 80:71–84

Warwick SI, Simard MJ, Légère A, Beckie HJ, Braun L, Zhu B, Mason P, Séguin-Swartz G, Stewart CN Jr (2003) Hybridization between transgenic *Brassica napus* L. and its wild relatives: *B. rapa* L., *Raphanus raphanistrum* L., *Sinapis arvensis* L. and *Erucastrum gallicum* (Willd.) O.E. Schulz. Theor Appl Genet 7:528–539