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Expression of GFP and Bt transgenes in *Brassica napus* and hybridization with *Brassica rapa*

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Abstract It is possible to monitor the movement of transgenes by tagging them with green fluorescent protein (GFP). In order to develop a model to study transgene flow, canola (*Brassica napus* cv Westar) was transformed with two GFP constructs, *mGFP5er* (GFP only) and pSAM 12 [GFP linked to a synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin gene]. Transformed callus sectors that fluoresced green were preferentially selected in the tissue culture process. Four independent GFP canola events and 12 events of GFP/Bt canola were regenerated through tissue culture. GFP fluorescence was macroscopically detectable throughout the entire life cycle of canola. The GFP/Bt events were insecticidal to neonate corn earworm (*Helicoverpa zea*) larvae and prevented herbivory damage. Fluorescence intensity at 508 nm varied between the independent transformation events, and ranged from 7.6×10^5 to 13.8×10^5 (counts per second) in contrast with the wild-type at 5.3×10^5 cps. Nine GFP/Bt and three GFP events were hybridized with three wild accessions of *B. rapa*. The resultant hybrids fluoresced green and were insecticidal to neonate corn earworm larvae to the same degree as the transgenic canola parents. However, fluorescence intensities of the hemizygous F_1 hybrid lines were lower than the respective original homozygous canola parents. Each F_1 hybrid line was backcrossed by hand onto the *B. rapa* parent, and transgenic backcrosses were produced at rates ranging from 15% to 34%. These data suggest that GFP can be used as a tool to monitor transgene flow from crop species to wild relatives.

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Introduction

Genetic modification of crops is rapidly becoming the technique of choice for the production of new agricultural varieties. The potential benefits range from reduced pesticide use (Stewart et al. 2000) to improved nutritional properties of food (DellaPenna 1999). However, effective application of this technology requires an ability to evaluate and understand potential complications. One such concern involves the transfer of fitness-enhancing transgenes from crop species to wild relatives. Gene flow between domesticated and wild species occurs in areas where sexually compatible species are present. Although this has always occurred in conventional agriculture, plant genetic engineering adds the complexity of novel transgene flow. Because of the potential consequences to the environment and agriculture, it is important to develop a system to monitor and evaluate the factors involved in transgene escape.

Analysis of gene flow with conventional molecular techniques is not practical in most agricultural situations. Techniques such as the polymerase chain reaction (PCR) and protein-blot analysis require tissue collection and time-consuming laboratory analysis, making them impractical to assess large-scale gene flow. Green fluorescent protein (GFP) can replace conventional methods and serve as an in vivo real-time marker for the presence of transgenes (Stewart 1996a, b). GFP was cloned from the jellyfish, *Aequorea victoria*, and has been expressed in many different organisms (Prasher et al. 1992; Chalfie et al. 1994; Leffel et al. 1997). When coupled to another transgene, GFP can indicate the presence of transgenic material by whole-plant fluorescence without destructive tissue sampling or laboratory analysis (Leffel et al. 1997). GFP could facilitate the monitoring of gene flow from agriculture to the environment, which would be intractable by conventional molecular techniques. For this

study, the *mGFP5er* gene has been linked with the synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin gene in a single plasmid (Haseloff et al. 1997). This construct was transferred into tobacco (*Nicotiana tabacum* cv Xanthi), and the resulting plants produced both proteins and exhibited both phenotypes (Harper et al. 1999).

The first goal of this project was to produce multiple transgenic events of GFP/Bt and GFP canola. GFP has been shown to be visible in developing callus, and GFP fluorescence was used as a method to improve the efficiency of the selection process. Whole-plant fluorescence patterns for canola have not been described in the literature, and these observations are important as GFP fluorescence changes throughout the life cycle. To be a useful marker under field conditions, the location of observable fluorescence must be known for accurate transgene monitoring.

The second goal of this project was to hybridize and backcross the GFP/Bt and GFP canola with *Brassica rapa*, to assess transgene expression and determine the feasibility of monitoring gene flow using GFP. *B. rapa* is a common weed in over 50 countries, and is a serious weed of cereal crops in Canada, Lebanon, New Zealand and Tasmania (Holm et al. 1997). *B. rapa* (AA, 2n=20) has been proposed to be an ancestral parent of *Brassica napus* (AACC, 2n=38) (U 1935), and interspecific hybridization has been documented under laboratory (Mikkelsen et al. 1996b; Metz et al. 1997) and field (Jorgensen and Andersen 1994; Mikkelsen et al. 1996a; Scott and Wilkinson 1998) conditions. The third goal was to analyze fluorescence intensity in the hybrids in order to begin understanding the dynamics of transgene expression in a weedy genetic background. The final goal of the project was to determine if specific canola events differ in their ability to produce transgenic backcrosses.

Material and methods

Vectors

All vectors were constructed from the pBIN19 plasmid and contained an *nptII* cassette for selection in plants with kanamycin. The mGFP5er vector contained the *mGFP5er* gene under control of the cauliflower mosaic virus 35 S promoter and an NOS (nopaline synthase) terminator (provided by Jim Haseloff). The mGFP5er-Bt cry1Ac vector (pSAM12) contained the *Bt cry1Ac* gene (synthetic, codon-optimized, truncated, courtesy of Dow AgroSciences) and the *mGFP5er* gene in the independent cauliflower mosaic virus 35 S promoter and NOS terminator cassettes (Harper et al. 1999).

Tissue culture and plant transformation

The transformation and tissue culture regeneration system was based on an existing protocol (Stewart et al. 1996b). Seeds from *B. napus* cv Westar, were surface-sterilized in a 20% bleach solution for 5 min with vigorous shaking. The seeds were then germinated on MS basal medium (Murashige and Skoog 1962). After 7 days, the seedlings were collected and the hypocotyls were cut into 1–2-cm pieces. The hypocotyl sections were placed on MS basal medium with 1 mg l^{-1} of 2,4-D for 24 h to precondition the material. Hypocotyls were inoculated with an *Agrobacterium* solution (10^8 cells ml^{-1} in liquid MS basal medium with acetosyringone 0.05 mM) for 30 min and co-cultivated on solid MS basal medium with 1 mg l^{-1} of 2,4-D for 3 days. Plant tissue was moved to the same media containing 400 mg l^{-1} of timintin to kill the *Agrobacterium*, and 20 mg l^{-1} of kanamycin to select for transformed cells. After 7 days, the hypocotyls were transferred to basal medium containing 4 mg l^{-1} of 6-benzylaminopurine, 2 mg l^{-1} of zeatin, 5 mg l^{-1} of silver nitrate, and the above antibiotics, for organogenesis. The tissue was transferred after 7 days to basal medium containing 4 mg l^{-1} of 6-benzylaminopurine, 2 mg l^{-1} of zeatin, with antibiotics. The shoots were removed and placed on basal medium containing 0.05 mg l^{-1} of 6-benzylaminopurine plus antibiotics for shoot development. The shoots were placed on basal medium containing 0.1% indole burytic acid plus antibiotics to promote root development. After the development of roots, the regenerates were moved to soil and hardened off. The plants were grown in a growth chamber with a photoperiod of 16 h at 20°C, and allowed to mature.

Visual assays

Assays for GFP fluorescence were conducted during the transformation experiment and canola life cycle. Developing callus was screened weekly for fluorescent sectors with a hand-held, longwave ultraviolet light (Spectroline high-intensity longwave UV lamp, BIB-150P model, 350 nm). Fluorescent sectors were preferentially tracked through the tissue culture process. To characterize the macroscopic fluorescence pattern, seedlings, plants at the 4–8 leaf stage and flowering plants were viewed to visualize green fluorescence of transgenic plants compared with red fluorescent nontransgenics of the same ages. Photos were taken with a digital camera (Kodak DC285 zoom camera) with a yellow filter. Multiple ultraviolet lamps were added to provide enough fluorescence to be detected by the digital camera.

PCR analysis

PCR was used to confirm the presence of the transgene in the genome of the plants. Genomic DNA extraction was carried out according to Stewart et al. (1997). Specific DNA primers for a Bt fragment (Stewart et al. 1996a); bases 200–219 5′-ATTTGGGGAATCTTTGGTCC-3′ and bases 789–770 5′-ACAGTACGGATTGGGTAGCG-3′, were used to amplify the Bt transgene. The *mGFP5er* gene was amplified with specific DNA primers; bases 5′-tacccagatcaTATGAAGCGG-3′ and bases 5′-TTGGGATCTTTCGAAAGGG-3′. The PCR procedure was carried out according to Stewart et al. (1996a).

Protein-blot analysis

The protein-blot analysis was done according to Stewart et al. (1996b). An extraction buffer containing 0.1 N NaOH and 2-mercaptoethanol was added to 0.2 g of fresh plant matter. The tissue was then homogenized with a hand drill-driven micropestle using 1.5-ml microcentrifuge tubes, and incubated on ice for 30 min. The homogenate was neutralized with 1 M Tris-HCl, and centrifuged at 10,000 rpm for 7 min. The supernatant was then removed, and the total protein in each sample was determined by Bradford total protein analysis using BSA as a standard. For the blot, 20 µg from each sample was loaded into a 10% polyacrylamide gel along with serial dilutions of a Bt standard. Immunostaining was carried out according to Pratt et al. (1986). For the primary antibody wash, rabbit polyclonal anti-Bt serum (courtesy of Dow AgroSciences) and rabbit polyclonal anti-GFP (Clontech) was used, followed by goat anti-rabbit (Sigma). Rabbit anti-goat/alkaline phosphatase (Sigma) was used as the tertiary antibody. Bt and GFP were detected on blots by exposure to nitroblue tetrazolium/bromochloroindolyl phosphate.

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 in the leaf tissue. The oldest non-scenecing leaf at the 6–8 leaf stage was excited at 385 nm, and emission spectra were recorded from 420–600 nm. A fiber optic cable was used to provide the excitation light to leaf tissue in vivo, and then to collect the emission spectra with no damage to the plant. Intensity was measured at 508 nm (green light) in counts per second (cps). The 450-nm wavelength, outside the GFP fluorescence spectrum, was set as the anchor for each sample. All samples were standardized to the average 450-nm value of non-transgenic canola to control for baseline variation.

Insect bioassay

Two 1-inch leaf discs were removed from the oldest non-senescing leaf on each plant, and were placed on moist filter paper in a Petri dish. Five neonate corn earworm (*Helicoverpa zea*) (CEW) larvae were placed on each leaf disk. The larvae were allowed to consume the plant material for 2 days under a 23-h photoperiod. The percent defoliation and the number of live insects were recorded at the end of the 2-day period.

Hybridization and backcrossing

Nine T_2 GFP/Bt events (GT 1–9) and three GFP events (GFP 1–3) were hand-crossed with three lines of *B. rapa* (from Irvine, Calif., USA, courtesy of Art Weiss; Milby, Quebec, Canada, and Waterville, Quebec, Canada, courtesy of Suzanne Warwick). Parental canola lines were germinated on moist filter paper, and fluorescent individuals were selected for the hybridization experiment. The *B. rapa* lines were used as the pollen recipients. Both species were allowed to flower, and hand-crossing was performed by removing a canola flower and pollinating the *B. rapa* plants. The hand-crossing continued as long as both plants continued to flower. All seeds were collected from the *B. rapa* parent, and were germinated on moist filter paper and screened by visual assay for GFP fluorescence. Plants that expressed GFP were backcrossed in the same fashion as above. The hybrids were used as pollen donors to produce backcrosses with their respective *B. rapa* accession.

Results

Transformation and tissue recovery

All tissue formed callus by the end of the 2nd week. Due to the relatively low concentration of kanamycin for selection of transgenic cells, callus formation occurred at a higher rate than was expected. Fluorescent sectors were detectable in the developing callus by the 3rd week post-coincubation (Fig. 1). The mGFP5er and pSAM 12 constructs generated 17 and 33 independent fluorescent sectors, respectively. The mGFP5er construct resulted in a higher percentage of fluorescent sectors compared to the pSAM 12 construct, at 47% and 10% respectively (Table 1). Hypocotyl sections containing fluorescent sectors were preferentially moved through the tissue culture process to overcome the low antibiotic selection pressure.

Shoots were selected from callus sections that contained fluorescent sectors. As shoots were produced, GFP fluorescence was detectable. All shoots were collected in order to determine the number of non-transgenic regenerates (escapes) compared to transgenic events. Six shoots were produced using the mGFP5er construct and 29 were produced using the pSAM 12 construct. Roots formed on shoots from both constructs, and four transgenic mGFP5er plants (labeled GFP 1 thru GFP 4) and 12 transgenic pSAM 12 (GFP/Bt) plants (labeled GT 1 thru GT 12) from independent transformation events (Table 1) were recovered. T_1 progeny of all transgenic events were germinated and scored for fluorescence, and resulted in 3:1 segregation ratios (transgenic:non-transgenic) suggesting single transgene insertion or multiple copies at a single locus (data not shown).

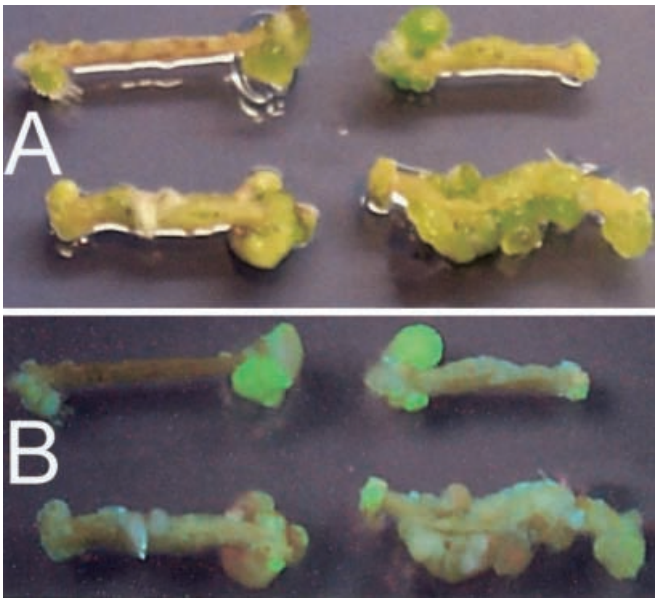
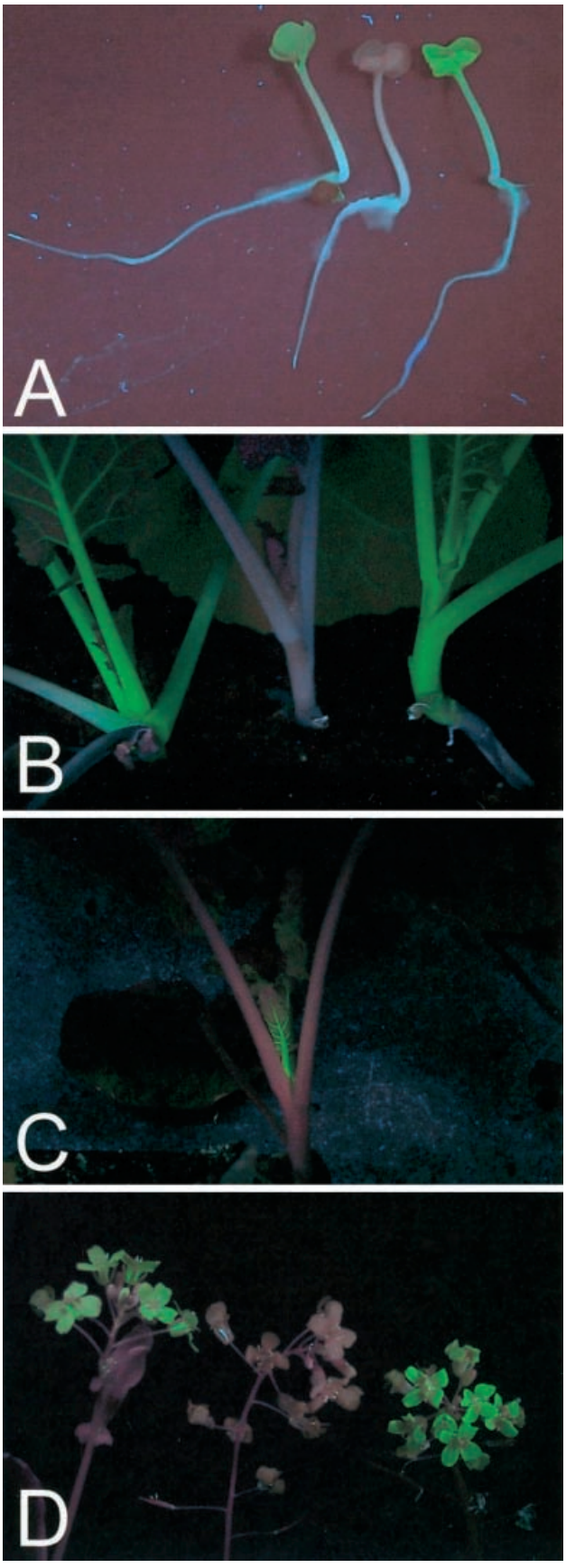


Fig. 1A, B GFP fluorescence in transformed canola callus. **A** Canola hypocotyl explants 4-weeks post coincubation with *Agrobacteria* containing *mGFP5er* under normal light. **B** The same tissue under UV light

Table 1 Summary of transformation efficiency using *Agrobacterium*-mediated transformation of one cultivar of canola (Westar) with two distinct plasmids

Construct	Start[a]	Sectors[b]	Shoots[c]	Transgenic[d]	Fertile[e]
mGFP5er	36	17	6	4	3
pSAM 12	337	33	29	12	11

[a] Number of hypocotyl segments co-incubated in *Agrobacterium* solution
[b] Number of GFP fluorescent sectors
[c] Number of shoots moved to rooting media
[d] Number of rooted shoots that tested positive for transgenicity
[e] Number of fertile transgenic plants determined by seed set



Visual assays

GFP fluorescence was detectable in all life phases of the T_1 canola plants. At the seedling stage, GFP was observable in the hypocotyls and cotyledons (Fig. 2A), and transgenic plantlets were selected at this stage. The roots of both transgenic and non-transgenic plantlets produced white auto-fluorescence, and, therefore, could not be used for screening purposes. As the plantlets developed, GFP fluorescence was best visualized at the apical meristem and in the vascular tissue (Fig. 2B). Non-transgenic material at this stage fluoresced light red, and was easily distinguished from the light to bright green of the transgenic plants. As the leaves developed and matured, the green fluorescence was less apparent and may have been masked by the increased red auto-fluorescence of chlorophyll. New growth at the meristem fluoresced brightly compared to older, mature leaves (Fig. 2C).

When the plant was at the end of its vegetative stage, it was difficult to differentiate between the leaves of the transgenic and non-transgenic as the result of the reddening of the GFP leaves by increased chlorophyll biosynthesis. The petals of the transgenic lines fluoresced bright green compared to the dull yellow of non-transgenic petals (Fig. 2D). GFP was macroscopically detectable at all stages of the canola life cycle, but the location of easily detectable fluorescence changed as the plant matured.

PCR analysis

DNA was collected from the selected T_1 progeny of each independent transformation event, and multiplex PCR was performed with Bt and GFP specific primers. Plants from the pSAM 12 construct (GT 1–12) produced 400- and 550-bp bands demonstrating that the *mGFP5er* gene was present and coupled to the Bt gene in the transgenic canola lines (Fig. 3). Plants produced by the mGFP5er construct produced a 400-bp band, illustrating that only the GFP gene was present. No positive PCR products were produced from non-transgenic Westar or the progeny of regenerates that did not fluoresce green.

Protein-blot analysis

Protein was extracted from the selected T_1 plants and hybrids, and characterized by protein blotting and immuno-

Fig. 2A–D Macroscopic detection of GFP fluorescence in the life cycle of canola. **A** Germinated seedlings from two primary transgenic events (*left and right*) and a wild-type canola (*center*). **B** GFP/Bt canola stems and veins (*left and right*) with wild-type (*center*) under ultraviolet light from plants, 5 weeks post-germination. **C** The apical meristem continues to fluoresce green as the plant matures, 8 weeks post-germination. **D** GFP/Bt flowers (*left and right*) with wild-type (*center*) under ultraviolet light from T_1 plants, 12 weeks post-germination

Table 2 Recovery of transgenic progeny from canola-*B. rapa* hybridizations and hybrids backcrossed into *B. rapa*. The numbers indicate the percent of progeny recovered from the handcrosses that screened positive for GFP fluorescence under ultraviolet light. Each handcrossed event resulted in the recovery of transgenic progeny, demonstrating that GFP can be used to follow transgene movements from crops plants to wild relatives. ANOVA indicates the GFP 3 line produced a significantly lower percentage ($p<0.05$) of transgenic BC_1 hybrids than four other lines (GT 1, GT 2, GT 5, GFP 1), however, the other 11 lines were not significantly different from one another. GT (canola events with GFP and Bt), GFP (canola events of GFP only), CA (California variety of *B. rapa*), QB1 (Milby, Quebec variety of *B. rapa*), QB2 (Waterville, Quebec variety of *B. rapa*)

Transgenic event	F_1 Hybrids				BC_1 Hybrids			
	CA	QB1	QB2	Total	CA	QB1	QB2	Total
GT 1	69%	81%	38%	62%	34%	25%	41%	33%
GT 2	38%	88%	81%	77%	23%	35%	31%	30%
GT 3	81%	50%	63%	65%	24%	10%	30%	20%
GT 4	38%	56%	56%	50%	7%	30%	36%	26%
GT 5	81%	75%	81%	79%	39%	17%	39%	31%
GT 6	50%	50%	54%	51%	26%	12%	26%	21%
GT 7	31%	75%	63%	56%	30%	19%	31%	26%
GT 8	56%	75%	69%	67%	22%	22%	21%	22%
GT 9	81%	31%	31%	48%	27%	28%	23%	26%
GFP 1	50%	88%	75%	71%	18%	33%	32%	27%
GFP 2	69%	88%	100%	86%	26%	20%	57%	34%
GFP 3	19%	38%	19%	25%	10%	22%	11%	15%

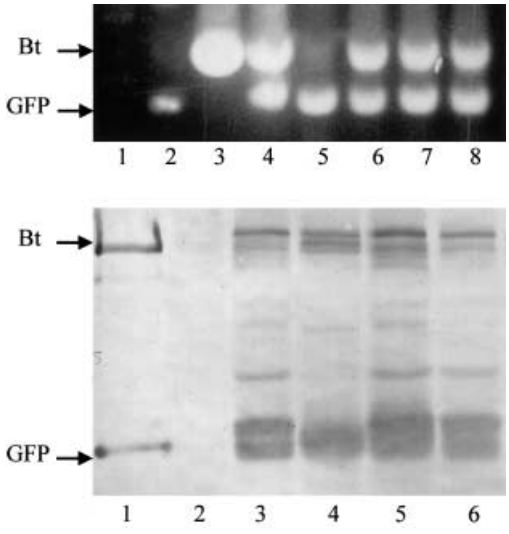


Fig. 3 Molecular analysis of GFP/Bt canola. *Top* PCR confirms the presence of GFP and Bt genes. *Lane 2* contains the *mGFP5er* plasmid, and *lane 3* is an amplified Bt gene. *Lane 4* contains the *pSAM 12* construct, which couples the GFP and Bt genes. *Lane 5* is a GFP transgenic canola event (GFP 1), and *lanes 6–8* are GFP-Bt events (GT 1, GT 2 and GT 3). *Lower* Western-blot analysis confirms expression of Bt and GFP proteins in transgenic canola. *Lane 1* contains Bt (60-kDa) and GFP (27-kDa) protein standards. *Lane 2* is a negative control (non-transgenic Westar). *Lanes 3–6* contain GFP-Bt canola extracts (GT 7)

staining. Transgenic plants produced from the pSAM 12 (GT 1–12) construct exhibited the presence of 60- and 27-kDa bands that corresponded to the purified Bt and GFP protein standards (Fig. 3). Plant extracts from the mGFP5er construct (GFP 1–3) produced only a 27-kDa band that corresponded to the production of GFP. In the plant extracts, both the Bt and GFP bands ran slightly slower than bacterially produced protein standards, as the result of post-translational modification of the proteins *in planta*. Multiple bands were apparent below the 60- and 27-kDa bands in the transgenic plants, but were absent from the control-plant extracts. These bands may represent breakdown products of the target proteins detected by the immunostaining procedure. No protein bands were detected in protein extracts from control canola plants. The maximal GFP synthesis estimated through densitometric assay in the GFP/Bt canola events was 0.25% of total extractable plant protein; whereas the highest *Bt cry1Ac* synthesis was 0.075%.

Fluorescence spectrophotometry

Homozygous T_2 canola plants at the 8-leaf stage of each GFP and GT transgenic event were analyzed using fluorescence spectrophotometry (Fig. 4). Two GFP events (GFP 1 and GFP 2) exhibited the highest average 508-nm emission peaks at 13.8±2.3 and 11.8±4.1 respectively (all units in 10^5 counts per second). Events generated from the pSAM 12 construct exhibited a range of emission averages from 7.6±0.3 to 10.3±1.0 cps (10^5). The average 508-nm emission for non-transformed canola plants was 5.3±0.6 cps (10^5).

Insect bioassay

Insect bioassays were conducted on leaf disks taken from multiple lines of GT, GFP, the F_1 hybrid, and wild-type canola (Fig. 5). GT events suffered low average degrees of de-foliation ranging from 0.5±0.9% to 7.5±5.0% (GT 8 and GT 3, respectively). Control and GFP plants suffered from similar degrees of de-foliation damage ranging from 60±16% to 70±10%, and illustrate that GFP alone has no insecticidal characteristics. Visual GFP fluorescence of

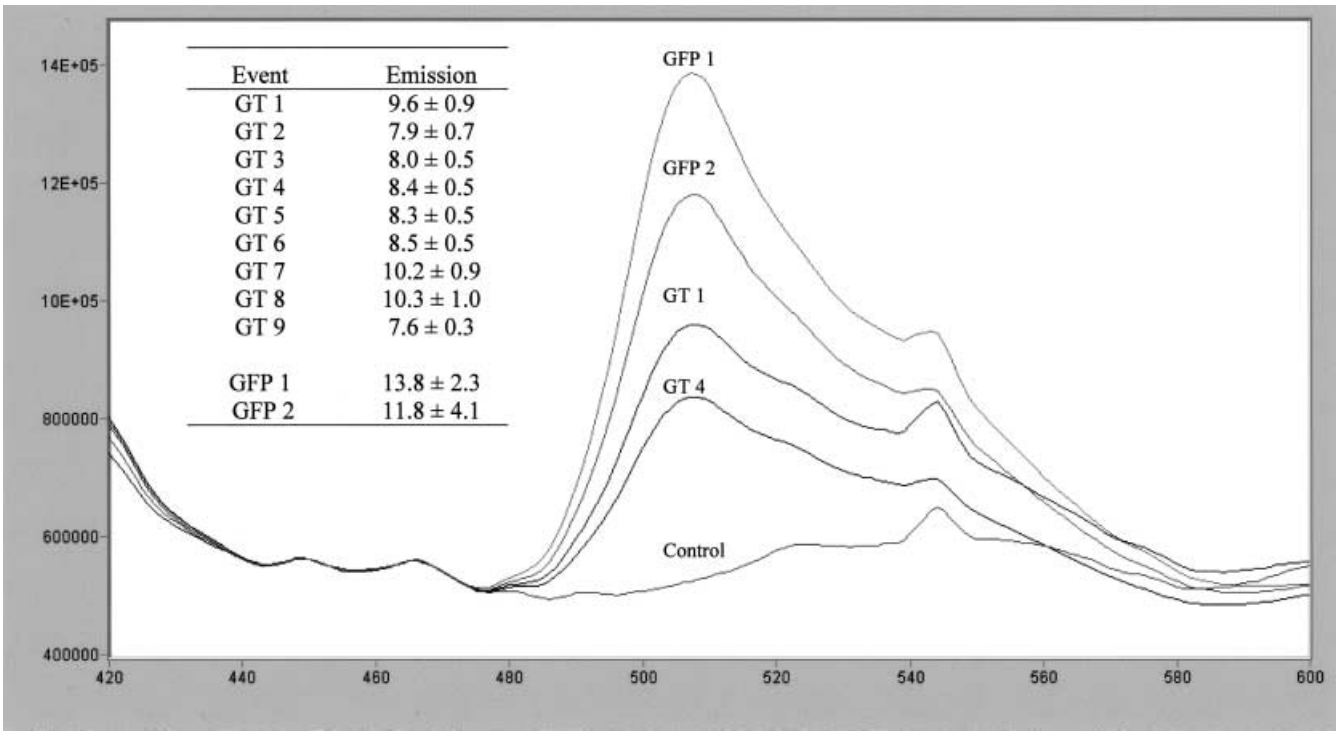


Fig. 4 Fluorescence averages for 11 independent GFP/Bt and GFP canola events. T_2 homozygous plants of each transgenic event were excited with 285 nm of UV light and scanned from 420 to 600 nm. The inset table shows the emission maximum at 508 nm (green light), and the emission intensity was recorded in counts per second (10^5)

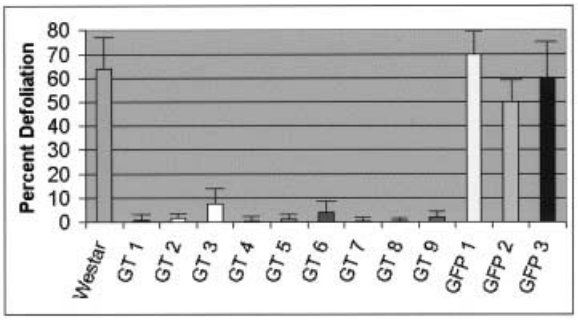


Fig. 5 Average insect herbivory on several GFP/Bt and GFP canola events. Non-transformed Westar and GFP canola events (GFP 1–3) suffered from heavy herbivory damage compared to the low de-foliation of GFP/Bt canola events (GT 1–9)

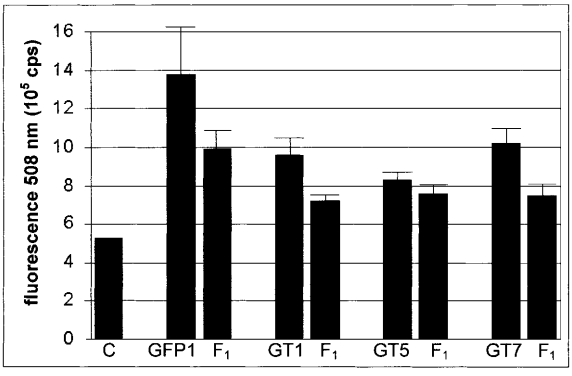


Fig. 6 Average fluorescence for four T_2 transgenic lines of canola and the resulting F_1 hybrids with *B. rapa*. Leaves were exposed to UV light and fluorescence measured at 508 nm (*t*-tests were used to determine differences in fluorescence intensity between the parental transgenic canola lines and the F_1 hybrids). In each case, the hybrids exhibited significantly less fluorescence than the parental line ($P<0.05$)

GT and F_1 hybrid plants was negatively correlated with leaf defoliation by CEW in the canola leaf samples.

Hybridization and backcrossing

All GT and GFP events generated F_1 transgenic hybrids through the hand-crossing experiment (Table 2). Plants in a portion of the canola lines (GT 1, GT 5, GT 7, GT 8, GFP 1 and GFP 2) were homozygous for GFP, while the other lines were still segregating (1:2:1). The hybrids were characterized in the same manner as the parental canola events, and demonstrated identical GFP macroscopic fluorescence patterns as the parent canola events (data not shown). The hybrid lines backcrossed with *B. rapa* resulted in a backcross generation (BC_1) for each line crossed (Table 2). The frequency of transgenic hybrids (F_1) recovered from each cross ranged from 19% to 100%, and the percent of BC_1 plants recovered ranged from 7% to 57% (Table 2).

Average GFP fluorescence intensity was significantly lower ($p<0.05$) in each hybrid line compared to the pa-

rental transgenic canola line (Fig. 6). In the case of GFP 1 canola, the difference in intensity at 508 nm was dramatically lower, an average of 13.8×10^5 (cps) for the canola versus 9.8×10^5 (cps) for the hybrids. However, in the case of GT 5, the difference was less extreme, an average of 8.3×10^5 (cps) for GT 5 versus 7.6×10^5 for the hybrids. In all cases, the hybrid fluorescence intensity was significantly lower ($p<0.05$), indicating an affect of transgene expression as the result of the transgene heterozygosity dosing effect.

Discussion

With the growing worldwide interest and concern over the production of genetically modified crops, systems that provide an ability to monitor gene flow may be valuable tools. Gene flow from conventional crops to wild relatives is a reality of modern agriculture, but has not been a concern because of a lack of identifiable complications and an inability to effectively monitor the situation. However, growing public confusion over GM foods has led to serious criticism of their safety including concern over the escape of novel transgenes that could result in unpredictable effects on the environment (Stewart et al. 2000). This study demonstrates that it is possible to use GFP to monitor the flow of transgenes, providing a tool to evaluate and address this concern. GFP/Bt canola will be useful because it combines the easy to use monitoring characteristics of GFP with an agronomically important transgene. These transgenic events will be used in field-hybridization studies to determine the feasibility of tracking transgenes under normal agricultural conditions.

GFP-aided selection

Several studies have used GFP as a selection agent in order to improve transformation efficiency (Elliot et al. 1998; Ghorbel et al. 1999; Tian et al. 1999; Vain et al. 2000). For example, GFP screening was used to enhance the efficiency of hygromycin selection for transgenic rice recovery (Vain et al. 2000). In this study, GFP fluorescence was used to increase the accuracy and predictability of the canola selection process. The use of GFP-aided selection resulted in a 4% transformation efficiency, which is consistent with previous canola transformation rates (Stewart et al. 1996b). Transgenic events produced consistent levels of protein production, in contrast to the variable production levels generated previously in non-GFP systems (Stewart et al. 1996b). This could be advantageous by facilitating the recovery of the highest-expressing transgenic events. GFP selection could be used to bypass much of the screening required to classify the expression level of primary transgenic events in current transformation systems, and GFP selection may be a feasible method to replace conventional antibiotic and herbicide selection regimes.

The pattern of macroscopic fluorescence in whole transgenic plants demonstrated in this study will facilitate the tracking of transgenic material under field conditions. The location of detectable fluorescence changes dramatically as canola progresses through its life cycle. The CaMV 35 s promoter produces high expression of GFP in young leaves and meristematic material, and GFP yields similar patterns as GUS under the control of identical promoters (Harper and Stewart 2000). When plants were at the 2–8 leaf stage, the leaves were green and distinguishable from wild-type. As the leaves matured, the green fluorescence was masked by the red autofluorescence of chlorophyll and could no longer be used for screening purposes. In mature plants, green fluorescence was detectable in meristems, such as nodes and flowers. An effective monitoring system must have a consistent pattern, and GFP fluorescence has been shown to be visible in young leaves, stems, veins and flowers.

Hybridization

Interspecific gene flow of a transgene was tracked in this study using GFP fluorescence. The production of GFP and GT canola will allow for the expansion of gene-flow studies to large agronomic plots. Large numbers of individuals may now be rapidly screened in situ, and the monitoring of transgenic material can be performed over many years without the destructive collection of tissue needed by traditional monitoring techniques. Also, hybridization events that occur at low frequencies and that exhibit subtle morphological changes in the progeny can be monitored by GFP fluorescence. GFP transgenic germplasm could revolutionize the understanding of gene flow in multiple plant genera, by illuminating complex events with a simple fluorescence profile.

The genomic location of transgene integration into canola, whether on the A or C genome, has been suggested to play a role in the ability of transgenic events to pass fitness-enhancing transgenes to *B. rapa* (Metz et al. 1997). In this model, hybrid plants were putatively triploid (AAC), and the chromosomes on the C genome were unstably passed or lost during meiotic divisions (Metz et al. 1997). If the transgene is on the C genome, the gene could be lost to the next generation leading to no transgenic backcrosses. By this model, the location of transgene insertion would result in different backcross frequencies between transgenic events, and certain lines would be “safer” in regards to gene flow and integration. This assumption has been challenged by the fact that the A and C genomes share a significant degree of homology, and recombination rates may be high and allow for increased rates of transgene integration into *B. rapa* (Tomiuk et al. 2000). The backcross frequencies presented in this study support the hypothesis that there are few likely “safe” locations in the canola genome with regards to gene flow. In this study, 12 independent

canola events generated backcrosses at similar rates. This is in contrast to the findings of Metz et al. (1997), in which two independent herbicide-tolerant canola events produced BC_1 plants at significantly different rates. The sample size of 12 transgenic events presented in this study is the largest analyzed, and adds data to an argument that has been historically theoretical. Further studies utilizing GFP canola and introgressed relatives in the *Brassiceae* family will expand the knowledge of gene flow in this agriculturally important group of crops and weeds.

Transgene expression

Differences in transgene expression in the background of weedy relatives compared to the original crop species have been demonstrated. The differences in fluorescence intensity detected between the F_1 hybrids and the parental canola lines could be attributed to several factors. The hybrid plants were hemizygous for the transgene, and other studies have detected differences in fluorescence intensity between homozygous and hemizygous individuals (Niwa et al. 1999; Molinier et al. 2000). In this study, the average fluorescence intensity of the F_1 hybrids was approximately half the magnitude of the homozygous canola lines. This is one of the first studies to quantify differences in fluorescence-maximum through fluorescent spectrophotometry, and zygosity dosage correlates with the magnitude of fluorescence. This result does not preclude an effect of the genetic background of the wild relative on transgene expession.

There are limitations to the GFP monitoring system presented in this study. In the pSAM 12 construct, the transgenes are simply linked in separate expression cassettes, and they might become unlinked over multiple generations through the process of recombination. Another limitation is the relative expression differences of the two transgenes. However, this study has shown that if high expressing events are selected in tissue culture, then both transgenic proteins will be expressed to a high degree. GFP in transcriptional or translational fusions could solve these potential limitations by generating a single GFP-Bt fusion protein. Additional research also needs to be performed to test the GFP monitoring system under field conditions. A GFP monitoring system utilizing either linkages or fusions is one of the best available technologies to facilitate the large-scale and long-term monitoring of fitness enhancing transgenes. GFP allows for the large and relevant field experiments that are warranted to assess the risks of potentially beneficial agricultural biotechnology.

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