ABSTRACT

HALFHILL, MATTHEW DAVID. Gene flow from transgenic crops to wild relatives. (Under the direction of Thomas W. Rufty and Arthur K. Weissinger)

Gene flow from transgenic crops to wild relatives resulting in transgenic hybrid populations is a possible outcome of the utilization of genetically modified crop varieties. 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). Nine GFP/Bt and three GFP transgenic events were hybridized and backcrossed two generations with three accessions of a wild relative, *Brassica rapa*. The resultant hybrids fluoresced green and were insecticidal to neonate corn earworm larvae to the same degree as the transgenic canola parents.

GFP fluorescence was analyzed during leaf development and was shown to be variable at each leaf position over time and among different leaves on the same plant. A leaf had its highest GFP fluorescence after emergence, and subsequently, its fluorescence intensity decreased over time. Younger leaves were significantly more fluorescent than older leaves on the same plant. The GFP fluorescence intensity was directly correlated with the concentration of soluble protein per unit wet mass. GFP fluorescence was correlated with Bt concentration.

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. 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). The GFP transgene was shown to demonstrate additive gene expression.

Two experiments were performed to evaluate the consequences of introgression of transgenes into hybrid generations on crop production. First, vegetative growth potential and nitrogen use efficiency was analyzed for transgenic hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk) along with transgenic *Brassica napus* (canola) and non-transgenic *B. rapa*. Under optimal conditions, *B. rapa* exhibited higher rates of growth when compared to canola and transgenic hybrid generations. Second, the competitive ability of transgenic hybrids, non-transgenic hybrids, canola and *B. rapa* when grown with a model crop, wheat (*Triticum aestivum*), was quantified by determining crop reduction under field conditions. Transgenic hybrids (BC_2F_2 Bulk) were the least competitive with wheat when compared to the other *Brassica* competitors (25.5% versus 47.1% crop reduction, respectively).

Gene flow of GFP and Bt transgenes was quantified under field conditions. Under a high crop to wild relative ratio (600:1), hybridization frequency with *B. rapa* differed among transgenic canola events (ranging from ca. 4% to ca. 22%), and demonstrated an average frequency over all events of ca.10%. Under a lower crop to wild relative ratio (180:1), hybridization frequency with *B. rapa* was consistent among the canola lines at ca. 2%. Backcrossing frequency between interspecific hybrids and *B. rapa* was determined in two field experiments where the transgenic hybrid to wild relative ratio was between 1:5 and 1:15. Seeds produced from maternal hybrid plants were ca. 45% and 50% transgenic backcrosses, respectively, and when the wild relative served as the maternal parent, backcrossing frequencies were low at 0.060% and 0.088%.

GENE FLOW FROM TRANSGENIC CROPS TO WILD RELATIVES

by
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PERSONAL BIOGRAPHY

Matthew David Halfhill was born in Elkhart, Indiana on February 5th, 1975. He attended Concord High School, and graduated in 1993. He received a Bachelors of Science, Magna cum laude, highest honors in biology from Butler University in 1997. Matthew married his wife Jessica on August 9th, 1997. The family soon moved to Greensboro, NC, and Matthew attended the University of North Carolina at Greensboro in the summer of 1998. He received a Masters in Science in biology in the spring of 2000. Matthew and Jessica moved to Burlington, NC in the summer of 2000, and he attended North Carolina State University in the department of crop science in the fall of 2000. In the summer of 2002, Matthew and Jessica moved to Knoxville, Tennessee.

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Chapter 1: Literature review

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Introduction

Transgenic crops are rapidly becoming a staple of modern agriculture. Since 1992, the USDA has deregulated fifty-eight transgenic crop events for commercial field release (APHIS Permits 2003, www.isb.vt.edu). Deregulated crop species include beet (*Beta vulgaris*), canola (*Brassica napus*), corn (*Zea mays*), cotton (*Gossypium hirsutum*), flax (*Linum usitatissimum*), papaya (*Carica papaya*), potato (*Solanum tuberosum*), rice (*Oryza sativa*), soybean (*Glycine max*), squash (*Cucurbita pepo*), tobacco (*Nicotiana tabacum*), and tomato (*Lycopersicon esculentum*). Despite the fact that over 3.5 trillion transgenic plants have been grown in the USA, there have been few detectable ecological consequences (Stewart et al. 2000). This is not to say that growing transgenic plants is completely risk-free.

Of the widely acknowledged risks of transgenic plants (Stewart et al. 2000), one of the main concerns of scientists and governmental regulators is gene flow and the resulting ecological consequences of transgenes being harbored in hybrid and wild relative populations. Transgene movement via hybridization between closely related species can be a mode of transgene flow directly into wild populations (Raybould and Gray 1993). If expressed in the genetic background of a weed species, a transgene could conceivably change the fitness of the weed in nature. In the worst-case scenario, the weed could become more invasive and competitive.

Intraspecific and interspecific hybridization

Intraspecific hybridization could occur when transgenic crops are grown in close proximity to non-transgenic varieties and landraces. Volunteerism and the agricultural practice of saving seed from the previous year's harvest could allow transgenic material to be unintentionally persistent. Extensive transgene flow between canola varieties has been detected in Canada resulting in the generation of volunteers that contain three types of herbicide resistance (Hall et al. 2000, Beckie et al. 2003). Crops such as corn and other grain crops that are wind pollinated have a potential to pass genes to adjacent conspecifics independent of whether the crop is GM or a conventional variety. This is a particular problem for organic farmers, who ensure that their products are not genetically modified and could potentially suffer economic losses if transgenic material is found in their harvests. Fitness-enhancing genes could be dispersed within the same species with no hybridization barrier, which could lead to slightly higher numbers of GM individuals than are expected by regulatory agencies. Transgenic crops could rapidly accumulate fitness enhancing traits (transgene stacking) that could lead to new and potential unintended problems.

Hybridization between closely related species can be a mode of transgene flow directly into wild populations (Raybould and Gray 1993, Ellstrand et al. 1999). Crop plants with weedy wild relatives are of particular concern. If expressed in the genetic background of a weed species, a transgene could increase the fitness of the weed in nature. In the worst-case, if perhaps unlikely scenario, the weed could become more invasive and competitive, and in a relatively short time cause damage in natural ecosystems.

Several circumstances are required to allow gene flow between related species. The crop must have a naturally occurring wild relative growing near cultivation. Crops such as

corn and soybean have no relatives in the US and Canada; therefore, they represent no risk of interspecific gene flow. Alfalfa, *Brassica* crops, and rice are examples of crop species that do have wild relatives near cultivation (Raybould and Gray 1993, Warwick et al. 1999), and these species complexes should be the focus of future gene flow studies. The two species must share a degree of sexual compatibility, and distantly related species sometimes share enough genome homology to produce viable progeny (Scheffler and Dale 1994). The species must occur sympatrically, or at close enough distances to allow the transfer of viable pollen. Flowering of the cultivated and wild species must occur concurrently, in order for the two species to be interfertile. Many weeds have complex patterns of dormancy, asynchronous germination, and germination signaling that have been lost in crops by artificial selection (Adler et al. 1993, Linder 1998), and these factors may increase the period over which hybridization could occur.

The variable homology of the genomes between related species leads to a wide range of possibilities for the rate of introgression of a transgene, or any other gene, after the F_1 hybrid generation. Meiotic abnormalities caused by the distant relation between parental genomes can lead to decreased rates of introgression into new genotypes (Scheffler and Dale 1994, Jorgensen et al. 1996). Chromosomes can be lost or disrupted due to unequal pairing at metaphase, which results in higher rates of infertility and decreased rates of seed production. Recombination, an important process in the incorporation of foreign DNA, is diminished in the unstable chromosome configurations of hybrids from distant relatives. In contrast, hybrids produced by closely related species have been shown to combine fitness indices (seed production, pollen fertility, biomass, etc) that parallel the parental species (Metz et al. 1997, Hauser et al. 1998,a, Hauser et al. 1998,b). In this situation, the

hybridization barrier between these species can be very low, and the introgression of a transgene is likely. The reproductive fitness of interspecific hybrids affects the ability of a transgene to be lost in the genetic background of a wild relative.

The possibility for increased fitness of transgenic hybrids and backcrosses depends on the nature of the transgene and the environment (Ellstrand et al. 1999). For example, weeds containing a transgene that confers resistance to an herbicide would be a nuisance to agriculture, but would have little affect in a non-agricultural environment where the herbicide is absent. In contrast, an insecticidal *Bacillus thuringiensis* (Bt) transgene in a weed host could alter natural ecology by giving transgenic weeds a selective advantage as the result of natural insect pressure (Stewart et al. 1997) if that specific insect was critical to limiting the survival of the weed. Transgenes that provide fitness enhancing characteristics under natural conditions have the greatest potential to disrupt the balance of established ecosystems.

Brassica **model for the analysis of the consequences of gene flow**

Canola (*Brassica napus*) as a model to study the consequences of transgene flow has become increasingly relevant as the adoption of transgenic canola varieties has increased in the past 3 years (33% of the canola acreage in US and 70% in Canada is transgenic-- mainly for herbicide tolerance). During the period, Calgene (Monsanto) has tested Bt transgenic canola in ND, MN, and CA, and AgrEvo (Aventis) has performed trials in CA. In addition, Bt transgenic lines have been tested in Canada and India. In total, 172 experimental transgenic insect resistance trials with various crops have been performed in the US (APHIS Field Release Database; http://gophisb.biochem.vt.edu/).

Canola (oilseed rape) is an ideal crop for the study of transgene escape into wild relatives. Canola is an allotetraploid (AACC, 2n = 38) and has many wild weedy relatives such as birdseed rape (*Brassica rapa*, AA, 2n = 20) and wild radish (*Raphanus raphanistrum*, RrRr, 2n = 18) persisting in or near areas of cultivation. Birdseed rape is a common weed in many places where canola is grown (Holm et al. 1997), and wild radish is a noxious cosmopolitan weed that can exist outside the agricultural setting. These weeds have been shown to hybridize with canola under both laboratory and field conditions (Eber et al. 1994, Jorgensen et al. 1994, Lefol et al. 1996, Mikkelson et al.1996, Metz et al. 1997, Darmency et al. 1998, Chevre et al. 2000,). Also, canola transformation protocols have been developed and transgenic varieties have been widely studied under field conditions (Paul et al. 1995, Stewart et al. 1997, Darmency et al. 1998, Ramachandran et al. 2000). Canola has been transformed with fitness enhancing transgenes such as herbicide, disease, and insect resistance (Stewart et al. 1996, Metz et al. 1997, Harper et al. 1999, Halfhill et al. 2001).

It is generally accepted that transgenes will move from canola to *B. rapa*. Transgenic hybrids have been produced between transgenic canola modified with herbicide resistance genes and *B. rapa* (Mikkelsen et al. 1996, Metz et al. 1997). After one backcross generation, many of the progeny were morphologically and cytologically similar to the *B. rapa* parent (Metz et al. 1997). After successive backcrosses into the weedy parent, it was found that 10% of the subsequent BC3 and BC4 hybrids had resistance to the herbicide (Metz et al. 1997). This illustrates that transgenes can be passed between species and continue to be expressed in successive generations. Few studies have been performed to produce transgenic hybrids that would have a putative selective advantage outside agriculture.

Transgenic interspecific hybrids have been produced involving transgenic canola modified with herbicide resistance genes with wild *B. rapa* (Mikkelsen et al. 1996, Metz et al. 1997). After one backcross generation, many of the progeny were morphologically and cytologically similar to the *B. rapa* parent (Metz et al. 1997). After successive backcrosses into the weedy parent, it was found that, as expected, up to 50% of the subsequent BC3 and BC4 hybrids had resistance to the herbicide (Metz et al. 1997). This illustrates that a transgene can be passed between species and expressed in successive generations.

Transgene flow from canola to wild radish (*Raphanus raphanistrum*) occurs at much lower levels. Depending on the wild radish genotype, the cross can occur in the field between 1 in 198,084 crosses (Chevre et al. 2000). In Australia, the number rose to 1 in 25 million (Rieger et al. 2001). The fertility of the hybrids was also diminished. However, given the nature of the severity of wild radish as a weed it seems prudent to also assess the weediness potential of insecticidal transgenic wild radish. Wild radish has much less probability of gene transfer from canola, but if it does receive a transgene, it could be a greater problem because of its ecology and increased competitiveness. The canola/weed system continues to be a viable worse-case scenario for agricultural gene escape.

GFP as a monitor for transgene escape

Monitoring transgenes in nature with conventional molecular techniques has been difficult and time intensive. Polymerase chain reaction (PCR), protein blot analysis, and Beta-glucuronidase (GUS) require destructive tissue sampling, expensive substrates (X-Gluc in the case of GUS), and are time intensive. Green fluorescent protein (GFP) can replace these methods and be an *in vivo* real-time marker for the presence of transgenes (Stewart

1996). When coupled to a fitness enhancing transgene of interest, GFP can indicate the presence of transgenic material by whole plant fluorescence without destructive tissue sampling and the addition of expensive substrates (Chalfie et al. 1994, Harper et al. 1999). GFP will facilitate the tracking of escaped transgenes in the natural environment, which would be almost impossible by conventional molecular techniques.

With the practical limitations of molecular monitoring techniques, GFP monitoring offers an easy-to-use, noninvasive alternative to track transgenes on a large scale (Stewart 1996). GFP was first cloned in 1992 from the jellyfish *Aequorea victoria*, and has since been cloned in many different organisms (Prasher et al. 1992, Chalfie et al. 1994). The GFP gene codes for a 27 kDa protein that fluoresces green when excited by ultraviolet (360-400 nm) or blue (440-480 nm) light. The protein is composed of 238 amino acids, and forms the shape of cylinder consisting of 11 β-sheets with connecting α-helixes segments. In the center of the "β-can", the chromophore resides as part of an α-helix and is composed of three residues; serine65, tyrosine66, and glycine67 (Yang et al. 1996). Molecular oxygen forces the bonding of Ser 65 and Gly 67 to complete the imidazalone ring of the mature chromophore (Yang et al. 1996). GFP is very stable over a wide range of conditions, and is resistant to many proteases. GFP is an ideal marker for transgenic material due to its fluorescence and stable structure.

The native GFP gene has been optimized for brighter fluorescence in plants (Haseloff et al. 1997, Chiu et al. 1996, Siemering et al. 1996). In its native form, the GFP gene contains a cryptic intron that is spliced when the mRNA is processed leading to poor fluorescence. Modifying the codon usage to avoid the recognition of the cryptic intron in the plant restored fluorescence (Haseloff et al. 1997). Endoplasmic reticulum targeting

sequences have been added to the gene, resulting in protein concentration within the cell and limiting possible toxic effects of the protein (Haseloff et al. 1997). In addition to containing the above modifications, the *mGFP5er* gene also has two mutations (V163A and S175G) that enhance folding of the protein at higher temperatures (Siemering et al. 1996). This gene has been tested in tobacco (*Nicotiana tabacum*) under field conditions, and the plants synthesized the protein and remained fluorescent throughout the growing season (Harper et al. 1999). The *mGFP5er* gene has been shown to be a feasible transgene monitor in plants under field conditions (Harper et al. 1999).

Objectives

In addition to the prevalence and increasing popularity of transgenic canola as a crop, the issue of gene flow and ecological consequences in transgenic weeds has become more prominent. While the acceptance of transgenic crops has not been accepted globally, there has been increasing support to assess the field and ecological performance of transgenic varieties around the world. In Europe, Canada, Australia, and the US, canola receives attention because of the cosmopolitan problem of compatible weedy species. It is also generally regarded that there will be gene flow from canola to *B. rapa* in all these locations. This research will explore six objectives to expand the knowledge of hybridization frequency, transgene expression and detection, and the consequences of gene flow to wild populations.

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

2. **Instrumentation and methodology for quantifying GFP fluorescence in intact plant organs**

3. **Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during plant development in transgenic canola, *Brassica napus* L.**

4. **Additive transgene expression and genetic introgression in multiple green fluorescent protein transgenic crop x weed hybrid generations**

5. **Altered vegetative growth and competitiveness associated with gene flow from transgenic *Brassica napus* (canola) to weedy *B. rapa***

6. **Gene flow from transgenic *Brassica napus* L. and crop/weed hybrids with relative weeds under field conditions**

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Chapter 2: 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 twelve 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 *Brassica 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.

Key words: transgenic canola, GFP, interspecific hybridization, *Brassica rapa*, *Bacillus thuringiensis*

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 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, 1996). GFP was cloned from the jellyfish, *Aequorea victoria*, and has been expressed in many different organisms (Chalfie et al. 1994, Leffel et al. 1997, Prasher et al. 1992). 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 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. *Brassica 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). *Brassica rapa* (AA, 2n = 20) has been proposed to be an ancestral parent of *B. napus* (AACC, 2n = 38)(U, 1935), and interspecific hybridization has been documented under laboratory (Mikkelsen et al. 1996, b; Metz et al. 1997) and field conditions (Jorgensen and Andersen 1994, Mikkelsen et al. 1996, a; Scott and Wilkinson 1998). 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 last goal of the project was to determine if specific canola events differ in their ability to produce transgenic backcrosses.

Materials and methods

Vectors

All vectors were constructed from the pBIN19 plasmid and contained an *npt II* cassette for selection in plants with kanamycin. The mGFP5er vector contained the *mGFP5er* gene under control of the cauliflower mosaic virus 35S 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 independent cauliflower mosaic virus 35S 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. 1996, b). Seeds from *Brassica napus* cv Westar, were surface sterilized in a 20% bleach solution for 5 minutes 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} 2,4-D for 24 hours to precondition the material. Hypocotyls were inoculated with an *Agrobacterium* suspension (10^8 cells mL^{-1} in liquid MS basal medium with acetosyringone 0.05 mM) for 30 minutes and co-cultivated on solid MS basal medium with 1 mg L^{-1} 2,4-D for 3 days. Plant tissue was moved to new plates of the same media containing 400 mg L^{-1} timintin to kill the *Agrobacterium,* and 20 mg L^{-1} kanamycin to select for transformed cells. After 7 days, the hypocotyls were transferred to basal medium containing 4 mg L^{-1} 6-benzylaminopurine, 2 mg L^{-1} zeatin, 5 mg L^{-1} silver nitrate, and the above antibiotics for organogenesis. The tissue was transferred after 7 days to basal medium containing 4 mg L^{-1} 6-benzylaminopurine, 2 mg L^{-1} zeatin, with antibiotics. The shoots were removed and placed on basal medium containing 0.05 mg L^{-1} 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 hrs at 20^o 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, long-wave 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 non-transgenics 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. 1996, a): 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'. PCR procedure was carried out according to Stewart et al. (1996, a).

Protein blot analysis

The protein blot analysis was done according to Stewart et al. (1996, b). 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 minutes. The homogenate was neutralized with 1 M Tris-HCl, and centrifuged at 10,000 rpm for 7 minutes. The supernatant was then removed, and 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 as 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 & Glen Spectra, Edison, NJ, USA) with DataMax and GRAMS/386 software (Galactic Industries Corporation, Salem, NH, USA) was used to quantify GFP fluorescence in the leaf tissue. The oldest intact 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 intact 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 two days under a 23-hour photoperiod. Percent defoliation and number of live insects were recorded at the end of the two-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 *Brassica rapa* (from Irvine, California, 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 pollinating the *B. rapa* plants with pollen from a detached canola flower. 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 second week. Due to the relatively low concentration of kanamycin (20 mg L^{-1}) 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 third week post-coincubation (Figure 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 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: nontransgenic) suggesting single transgene insertion or multiple copies at a single locus (data not shown).

Visual assays

GFP fluorescence was detectable in all phases of the life cycle of the T_1 canola plants. At the seedling stage, GFP was observable in the hypocotyls and cotyledons (Figure 2, A), 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 (Figure 2, B). Nontransgenic 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 (Figure 2, C).

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 (Figure 2, D). 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 base pair bands demonstrating that the

mGFP5er gene was present and coupled to the Bt gene in the transgenic canola lines (Figure 3). Plants produced by the mGFP5er construct produced a 400 base pair band, illustrating that only the GFP gene was present. No positive PCR products were produced from nontransgenic 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 immunostaining. 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 (Figure 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 (Figure 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, F_1 hybrid, and wild type canola (Figure 5). GT events suffered low average degrees of defoliation 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 defoliation damage ranging from 60 ± 16% to 70 ± 10%, and illustrate that GFP alone has no insecticidal characteristics. Visual GFP fluorescence of GT and F_1 hybrid plants was negatively correlated with leaf defoliation by CEW in the canola leaf samples.

Hybridization and backcrossing

F_1 transgenic hybrids were produced from all GT and GFP events by hand-crossing (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 backcrossed 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 parental transgenic canola line (Figure 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 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 lead 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. 1998). For example, GFP screening was used to enhance the efficiency of hygromycin selection for transgenic rice recovery (Vain et al. 1998). 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. 1996, b). Transgenic events produced consistent levels of protein production, in contrast to the variable production levels generated previously in non-GFP systems (Stewart et al. 1996, b). 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 35s 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 likely few "safe" locations in the canola genome with regards to gene flow. In this study, twelve 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 (Molinier et al. 2000, Niwa et al. 1999). 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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Table 1. Summary of transformation efficiency using *Agrobacterium*-mediated transformation of one cultivar of canola (Westar) with two distinct plasmids. [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 the presence of the transgene. [e] Number of fertile transgenic plants determined by seed set.

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

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 eleven 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*).

	F_1 Hybrids				BC_1 Hybrids			
Transgenic event	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%

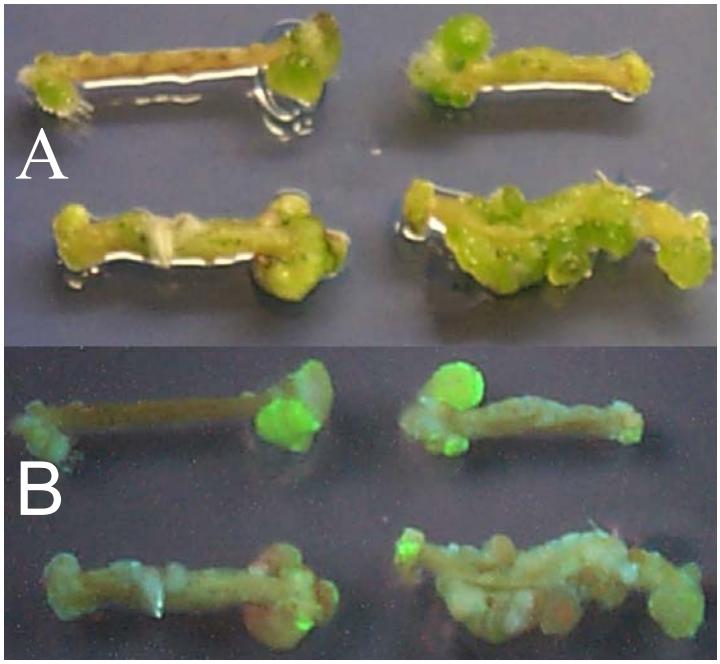

Figure 1. GFP fluorescence in transformed canola callus. A) Canola hypocotyl explants four weeks post coincubation with *Agrobacteria* containing *mGFP5er* under normal light. B) The same tissue under UV light.

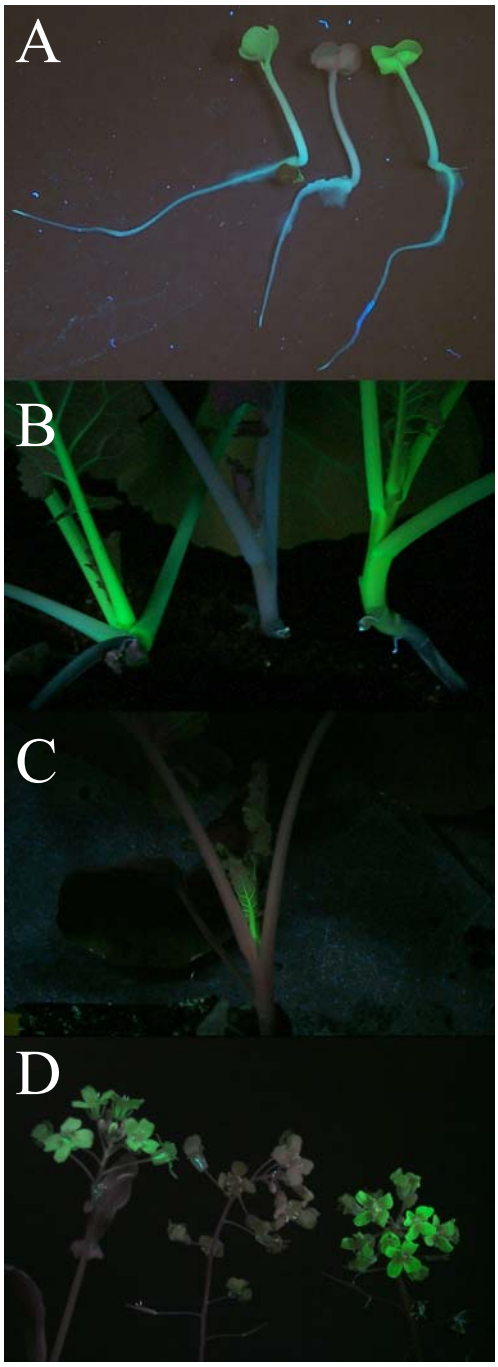

Figure 2. Macroscopic detection of GFP fluorescence in the life cycle of canola. A) Germinated seedlings from two primary transgenic events (left and right) and a wild type canola (center). B) GFP/Bt canola stems and veins (left and right) with wild-type (center) under ultraviolet light from plants, 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.

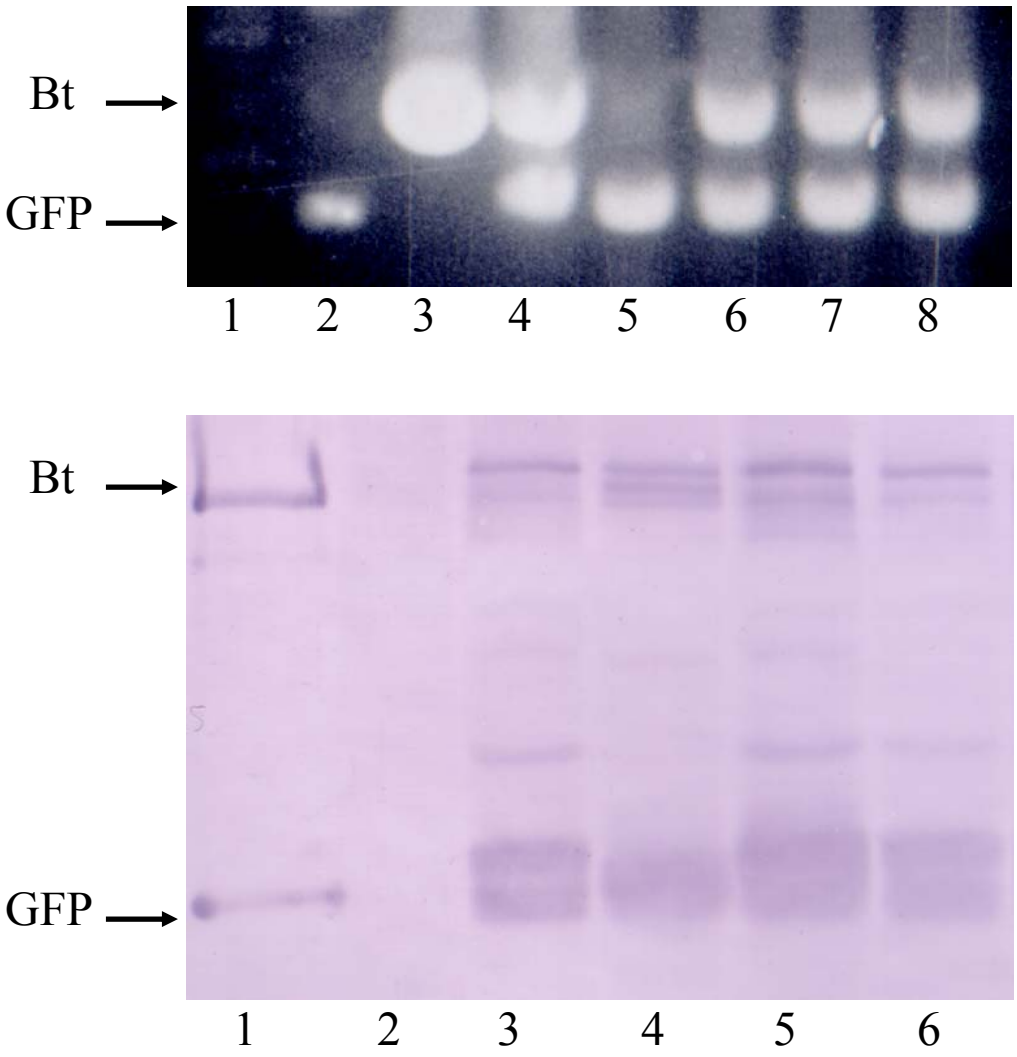

Figure 3. Molecular analysis of GFP/Bt canola. Top) PCR confirms the presence of GFP and Bt genes. Lane 2 contains the *mGFP5er* plasmid, and lane 3 is an amplified Bt gene. Lane 4 contains the *pSAM 12* construct, which couples the GFP and Bt genes. Lane 5 is a GFP transgenic canola event (GFP 1), and 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 contains GFP-Bt canola extracts (GT 7).

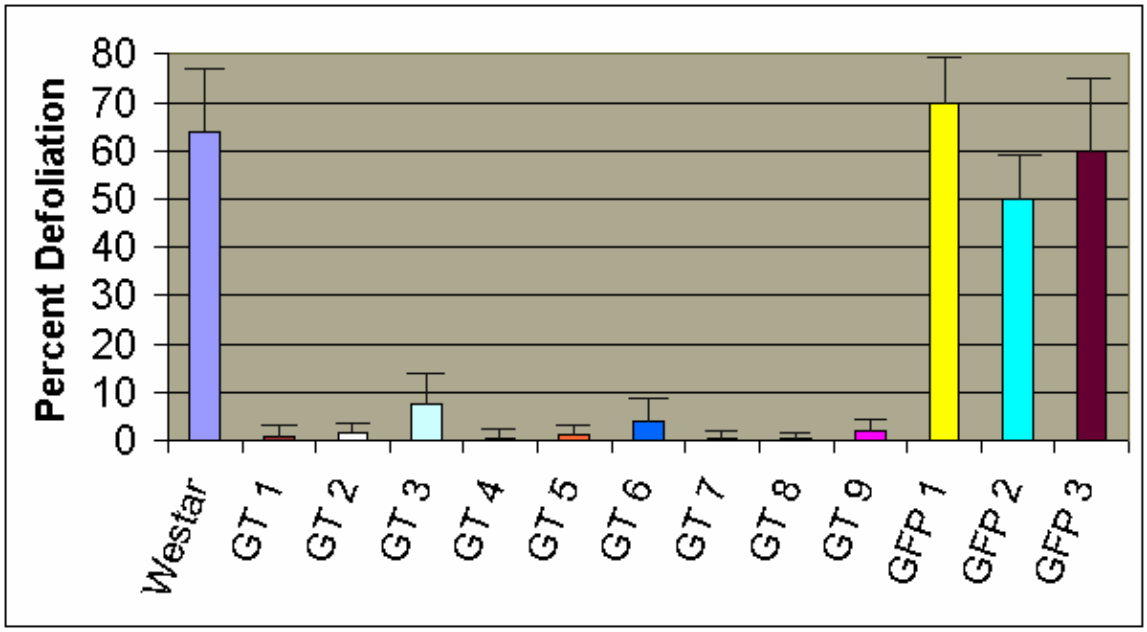

Figure 4. Average insect herbivory on several GFP/Bt and GFP canola events. Nontransformed Westar and GFP canola events (GFP 1-3) suffered from heavy herbivory damage compared to the low defoliation of GFP/Bt canola events (GT 1-9).

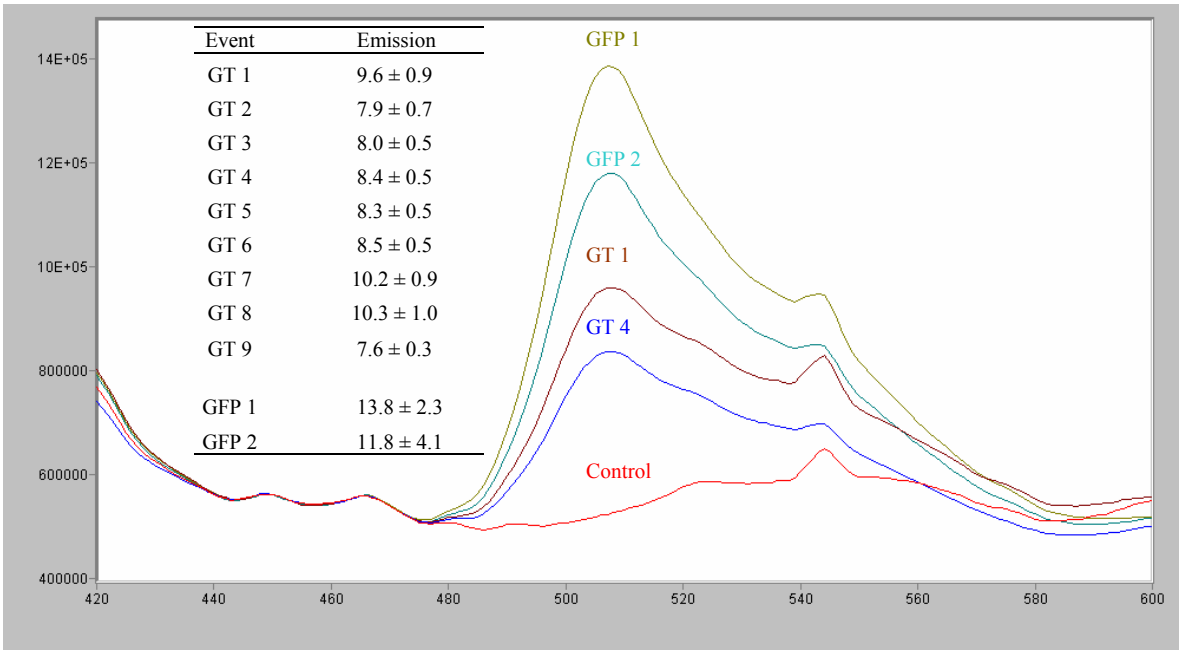

Figure 5. Fluorescence averages for eleven independent GFP/Bt and GFP canola events. T_2 homozygous plants of each transgenic event were excited with 385 nm UV light and scanned from 420-600 nm. The inset table shows the emission maximum at 508 nm (green light), and the emission intensity was recorded in counts per second (10^5).

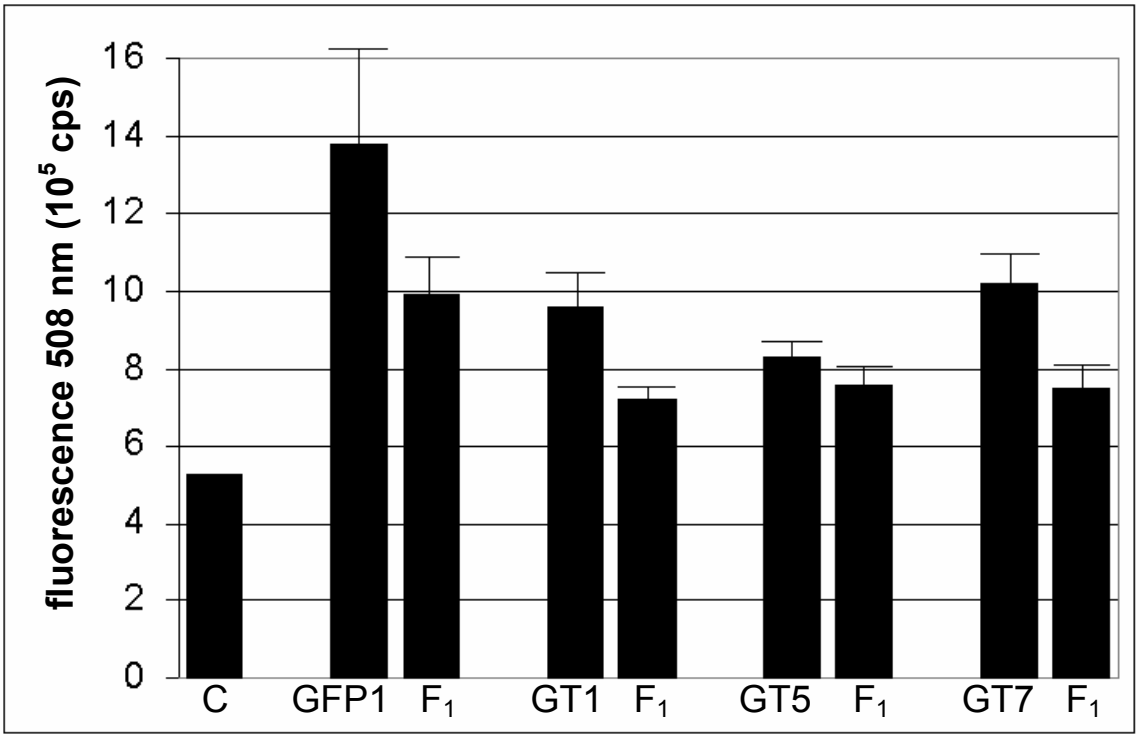

Figure 6. Average fluorescence for four T_2 transgenic lines of canola and the resulting F_1 hybrids with *B. rapa*. Leaves were exposed to UV light (385 nm) and fluorescence measured at 508 nm (ttests were used to determine differences in fluorescence intensity between the parental transgenic canola lines and the F_1 hybrids). In each case, the hybrids exhibited significantly less fluorescence than the parental line ($p < 0.05$).

Chapter 3: Instrumentation and methodology for quantifying GFP fluorescence in intact plant organs

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Abstract

The General Fluorescence Plant Meter (GFP Meter) (Opti-Sciences, Inc., Tyngsboro, MA, USA) is a portable spectrofluorometer that utilizes a fiber optic cable and a leaf clip to gather spectrofluorescence data. In contrast to traditional analytical systems, this instrument allows for the rapid detection and quantitative measurement of fluorescent proteins under field conditions with no damage to plant tissue. In this report, we discuss the methodology of gathering and standardizing spectrofluorescence data from tobacco and canola plants expressing a green fluorescent protein (GFP). Furthermore, we demonstrate the accuracy and effectiveness of the GFP Meter. We first compared GFP fluorescence measurements taken

by the GFP Meter to those taken by a standard lab-based spectrofluorometer, the Fluoro-Max2 (Jobin Yvon & Glen Spectra, Edison, NJ, USA). Spectrofluorescence measurements were taken from the same location on intact leaves. When these measurements were tested by simple linear regression analysis, we found that there was a positive functional relationship between instruments. Finally, to exhibit that the GFP Meter recorded accurate measurements over a span of time we completed a time-course analysis of GFP fluorescence measurements. We found that only initial measurements were accurate; however, subsequent measurements could be used for qualitative purposes.

Key words: transgenic, fluorescence, plants, gene expression, protein quantification, spectrofluorometer, portable device

Introduction

Fluorescent proteins are becoming a staple technology in plant genetic research. The green fluorescent protein (GFP), from the jellyfish *Aequorea victoria*, was the first in this category of genetic markers to be cloned (Prasher et al. 1992) and expressed in plant cells (Niedz et al. 1999). It has proven to be a powerful tool in plant research because it is a universal, *in vivo*, and a real-time transgenic marker (Haseloff and Amos 1995, Haseloff and Siemering 1998, Leffel et al. 1997, Prasher 1995, and Stewart 2001). However, under field conditions, optimal detection and measurement of GFP in intact leaves and other plant organs has not occurred due to the lack of appropriate and affordable instrumentation. This has limited researchers in their ability to collect data and poses a significant problem for future research.

There are several types of systems presently in use for the macroscopic detection and quantification of fluorescent compounds including: high-intensity UV lamps, spectrofluorometers (e.g., FluoroMax-2, Jobin Yvon & Glen Spectra, Edison, NJ, USA), and scanning laser systems (e.g., FluorImager, FluorImager SI, Molecular Dynamics, Sunnyvale, CA, USA). A hand-held 365 nm UV lamp, such as a UVP Model B 100 AP (UVP, Upland, CA, USA), allows for expeditious scanning of GFP fluorescence in whole plants. However, the UV light must be used in darkness, it is only effective for UV excitable GFP variants, and visual observation cannot be used to quantify GFP fluorescence. Spectrofluormeters and fluorescence imaging systems are capable of detecting the presence of GFP and also allow for quantification of fluorescent tissues (Leffel et al. 1997, Harper et al. 1999, Niwa et al. 1999, Halfhill et al. 2001). However, both systems are lab-based and expensive. For several years, plant researchers have sought a portable instrument that measures GFP in field plants under ambient lighting conditions. This paper describes such an instrument; the prototype Opti-Sciences General Fluorescence Plant Meter (GFP Meter). We also describe procedures to perform *in vivo* fluorescence measurements using laboratory equipment as controls.

Materials and methods

GFP Meter

The GFP Meter is a self-contained, field portable fluorescence detection and data logging instrument powered by an internal 1.2-ampere hour 12-volt gel lead acid battery (Fig. 1). A filtered light emitting diode (LED) generates excitation light when powered on. A small portion of this light is monitored to compensate for temperature drift. The output of the LED is focused onto one of three fiber ports. A driver, controlled by a micro-processing

unit (MPU), regulates the LED power level and compensates for changes in battery voltage. This excitation light travels through a band-pass filter to a fiber-optic cable and is then delivered to the sample. Attached to the end of this cable is a leaf clip, which has been installed to keep the cable in place. The light emitted from the sample enters back into the fiber optic cable and is directed through a band pass filter into a low noise preamplifier. This signal is then fed into an analog/digital signal processing and filtering unit slaved to the MPU. Fluorescence measurements appear in real time on a liquid crystal display in units of counts per second (cps). A 12-selection keypad provides user management of test functions and set-up. A non-volatile memory chip (capable of storing 1020 sample points) assures that data will not be lost when power is turned off or the battery removed and a RS-232 port enables downloading data to a computer. The GFP Meter uses a modulated detection system to minimize the effects of temperature drift and stray light. Virtually any band-pass filter combination can be used for excitation and emission. For this study, a 465 nm filter with a bandwidth of 35 nm was used for excitation. Channel 1 (GFP channel) used a 530 nm filter with a bandwidth of 35 nm for emission and channel 2 (chlorophyll channel) used a 680 nm filter with a bandwidth of 35 nm for emission. Chlorophyll data are not reported in this study.

FluoroMax-2

The FluoroMax-2 (Jobin Yvon & Glen Spectra, Edison, NJ, USA) is a lab-based spectrofluorometer system that utilizes a computer to process data. All FluoroMax-2 functions are under control of Datamax spectroscopy software (Galactic Industries Corporation, Salem, NH, USA). Light from a 150W xenon lamp enters an excitation

spectrometer, which delivers monochromatic light to a bifurcated fiber optic cable. A rubber protector surrounds the external end of the cable and extends 4 mm beyond to prevent signal disruption from dirt and debris and to provide a dark environment for measurements. Light flows through the cable only when a sample is being scanned. When scanning, the cable is then placed onto the sample and light emitted from the sample flows back through the fiber optic cable to the emission spectrometer where it is dispersed and directed to a signal photomultiplier detector. This fluorescence signal is then amplified and displayed on a computer monitor in units of counts per second (cps).

Plant material

Plants transgenic for GFP (*mGFP5er*) (Haseloff et al. 1997) and GFP/Bt (*mGFP5er /Bacillus thuringiensis*) under the control of constitutive *CaMV 35S* promoters were used in this study (Halfhill et al. 2001, Harper et al 1999). This GFP variant is excited equally by both blue (465 nm) and UV (395 nm) wavelengths. A total of twenty-six T_1 canola (*Brassica napus* cv. Westar) plants were grown in a plant growth chamber (Percival Scientific, Perry, Iowa, USA). The photoperiod for the chamber was set at 14/10 h light/dark and the temperature settings were 20/17 °C light/dark. Eight of these lines expressed GFP, and one was a control (wild-type) canola line (Halfhill et al. 2001). Plants were analyzed during the 12-15 leaf stage. Wild type and GFP tobacco (*Nicotiana tabacum* cv. Xanthi) plants were grown in the greenhouse and field (2). The greenhouse (Greensboro, NC, USA) temperature ranged between 26-30 °C and the plants were exposed to ambient light from March to August. Field plants were grown in a fine sandy loam soil at the Upper Piedmont Experimental Station, Reidsville, NC (36:23 N and 79:42 W) from June to August, 2001.

The plants were germinated in the greenhouse and transplanted in the field 105 days post germination. Both greenhouse and field plants were analyzed 124 days post germination. Plants grown in their respective areas were germinated the same day and were grown under identical conditions.

Fluorescent spectrophotometry

The GFP Meter and the FluoroMax-2 spectrofluorometer were used to measure GFP fluorescence of intact leaves. A spot 19.6 mm^2 on the underside of sample leaves and adjacent to the leaf mid-vein was excited at 465 nm with the GFP Meter and 385 nm with the Fluoromax-2. GFP Meter emission data was recorded at 530nm and Fluoromax-2 emission spectra was recorded from 420-560nm.

The FluoroMax-2 GFP fluorescence scans were standardized to control samples to account for baseline variation of each leaf measurement. The protocol for standardization involves selection of a wavelength outside the GFP fluorescence spectrum as a point of normalization for each FluoroMax-2 scan. For this study, the 450 nm wavelength was the anchor point. Emissions spectra were recorded from four individual control plants and averaged. Subsequently, each emissions scan was standardized to the average control for that species. Functionally, the 450 nm GFP value of the sample was subtracted from the 450 nm average control value. The resulting integer was then added to each wavelength value along the sample spectra (420-560 nm). This method eliminates differences outside the GFP emissions spectra allowing for comparison of GFP magnitudes. The GFP Meter did not have the option of standardization because the appropriate filter sets outside the GFP emissions range were not installed for this study.

Time course analysis

A time course study of GFP transgenic canola leaves was performed (Fig. 5) to analyze the stability and accuracy of GFP Meter measurements over time as compared with those of the Fluoromax 2. Measurements from both instruments were taken next to the mid-vein on the lower surface of a leaf. The fiber optic cables used to transport excitation and emission light to and from the instruments were kept in the same position for the duration of the experiment. This was consistent for all samples. These measurements were recorded every 15 seconds over a 450 second span. High and low expressing GFP canola leaves were used in this study.

Results and discussion

Standardization

Experimentation has demonstrated that plants from the same line exhibit baseline spectral differences when analyzed with FluoroMax-2 and excited with blue light (data not shown) and UV light (Fig.2). It is necessary to account for these differences so that the data are not misinterpreted. For example, field grown GFP tobacco plants that have similar GFP synthesis, measured by western blot analysis (data not shown), yield different GFP maximum values due to the baseline variation (Fig. 2, panel A). When the GFP spectra are standardized to the same 450 nm value (Fig. 2, panel B), the fluorescence maxima at 510 nm are similar for both samples. In addition, when these GFP spectral scans were compared to high-expressing plants (Fig. 2, plants 4), it appears that the low expressing plants (Fig. 2, plants 3) are synthesizing equivalent amounts of GFP (Fig. 2, panel C). After standardization (Fig. 2, panel D), these apparently similar spectral scans are sorted according to their

magnitude of GFP fluorescence. These data demonstrate that fluorescence scan standardization is necessary to obtain accurate measurements.

Performance of GFP Meter for intact leaf GFP measurements

Performance of the GFP Meter was compared to the Fluoromax-2, a validated GFP fluorescence measurement tool (Leffel et al. 1997, Harper et al. 1999, Halfhill et al. 2001). A comparison of instantaneous measurements from the GFP Meter (530 nm wavelength) and the Fluoromax-2 (standardized 510 nm wavelength fluorescence values) was completed to gauge accuracy. Even without standardization capabilities built into the GFP Meter, the regression analyses of growth chamber-grown GFP canola, greenhouse GFP tobacco, and field-grown GFP tobacco (Fig. 3, panels A, B, &C) produced high R^2 values (0.87, 0.88, and 0.89); indicates a positive functional relationship between instruments. However, incorporating a standardization feature into the GFP Meter may improve the instrument and produced higher R^2 values.

Time course analysis

Repeatability of the GFP Meter was important to analyze to ensure the instrument's readings were accurate over time. When we tested for repeatability of the FluoroMax-2 (repeated measurements, single spot on a leaf), we observed that there were no significant differences among measurements after standardization (data not shown). This was not the case with the GFP Meter (Fig. 4), where there was an obvious and unpredictable decrease in measurements over the course of time. After initial measurements, canola leaf sample fluorescence values demonstrated a sharp decline for the first 100 seconds, but stabilized for

the duration of the experiment. The same was true for tobacco (data not shown). This rate of decline was not consistent for each sample (Fig. 4). Therefore, after the initial measurement, any measurements taken thereafter from the same location cannot be compared quantitatively.

Optimizing the GFP Meter for use with GFP

There are two practical concerns that should be noted about the GFP Meter. The first concern involves baseline variation. Previously noted was that the FluoroMax-2 system experienced baseline variation among plants. In some cases, the FluoroMax-2 registered a 510 nm emission value for nontransformed plants that is similar to 510 nm fluorescence values for a plant expressing GFP (Fig. 2), and therefore, standardization is required. Consequently, if these values are not standardized a control plant may be mistaken as a plant expressing GFP. This baseline variation may be attributed cable angle deviation from perpendicular when taking measurements with the FluoroMax-2; the user imprecisely controls the angle at which the fiber optic cable is held. The angle at which the cable is held may influence the spectral scans causing them to migrate on the y-axis at the scan start point (Fig. 2, panels A and C). This may help to explain why the nonstandardized GFP Meter data exhibited such strong correlations to the standardized FluoroMax-2 data. The GFP Meter utilizes a leaf clip, which removes any chance of user variation because the fiber optic cable is held at a fixed angle. However, since it is not known if the GFP Meter exhibits some baseline variation the instrument may be improved by adding a standardization feature. More research is being performed to determine baseline variation.

The second concern involves comparing GFP Meter fluorescence values when the measurements are taken after the leaf clip has been on the sample for more than 15 seconds. Expression levels are incomparable if a reading is taken after this time due to an unpredictable decrease in fluorescence values (Fig. 4). This decrease may be due to photobleaching of GFP, but minimal photobleaching would occur if measurements were taken immediately. It is also possible that the modulation source is inducing kinetics as the graphed data points bore a distinct resemblance to a Kautsky curve (Kautsky and Hirsch 1931); which upon illumination of the plant material, there is a rapid rise in light emission from photosystem II fluorescence followed by a decline as it gradually settles out to a low steady state. Although expression levels cannot be directly compared, the GFP Meter can still be used for qualitative fluorescent protein detection purposes at any time.

Although these issues raise concerns about the effectiveness of the GFP Meter, they can be adequately addressed. In addition to the normal operating procedures outlined in the usage manual (provided by Opti-Sciences), the following methods should be performed to ensure the accuracy of GFP Meter measurements. An emission filter, outside the GFP fluorescence range, should be installed in channel 2 of the GFP Meter to control possible baseline variation. Channel 2 would then provide a value to be used for standardization to a control measurement. Furthermore, in order to minimize the effect of the decrease in fluorescence values over time, analysis should begin as soon as the leaf clip is placed onto the sample tissue. If these methods are followed, GFP expression levels can be compared between individual plants as well as among plant lines.

In summary, the GFP Meter was shown to be an effective tool for measuring the magnitude of GFP fluorescence in whole plant systems. Therefore, this instrument will

allow researchers to gather data quickly and accurately. Additionally, there are other benefits to the GFP Meter such as non-destructive plant tissue analysis. This manner of data collection allows for other ecological and physiological analysis to be performed on the transgenic material. Another benefit of this instrument is, its capability of employing different filter sets. This is encouraging, considering fluorescent proteins from other marine organisms have recently been cloned (Matz et al. 1999). If these fluorescent proteins are incorporated in plant genetic research the same way GFP has been then the potential utility of the GFP Meter will increase. Furthermore, as the use of fluorescent proteins in biological applications increase, so should the applications of the GFP Meter as a fluorescence detection and quantitation device.

Acknowledgements

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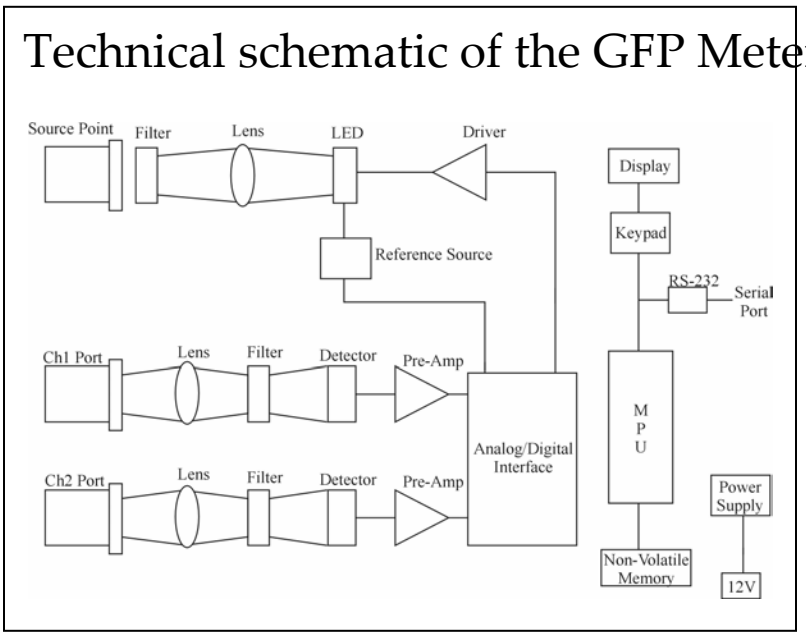

Figure 1. Technical schematic of the GFP Meter.

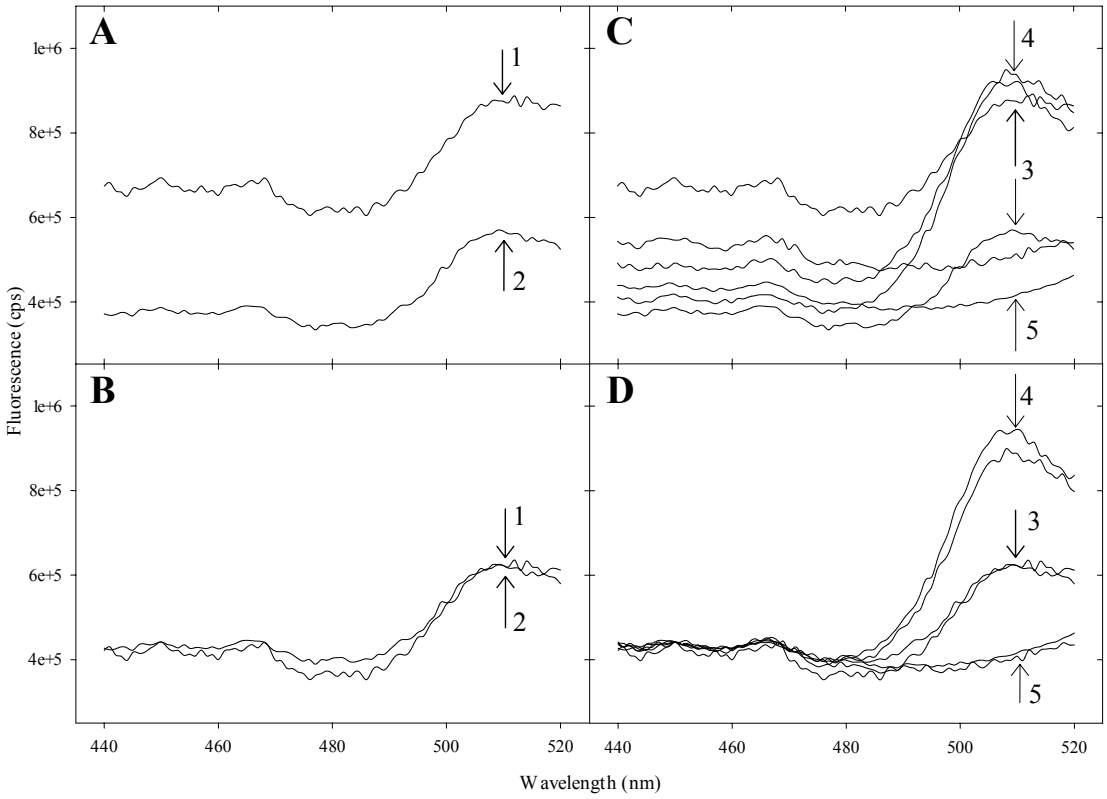

Figure 2. Standardization of spectrofluorometer (FluoroMax-2) measurements of field-grown GFP tobacco. Each line represents an individual tobacco plant. Panel A: two raw measurements (1 & 2) of similarly expressing GFP plants excited with 385 nm light; panel B: the same measurements as panel A, but standardized to the 450 nm emission wavelength of average non-transgenic controls (5) represented in panel D; panel C: raw measurements of high expressing (4), low expressing (3), non-transgenic tobacco (5; and panel D: the same measurements standardized to the 450 nm emission of average non-transgenic controls (5). In panel B, the arrows demonstrate the similarity of GFP fluorescence at 510 nm between the two plants when standardized. In panel D, standardization allows for the categorization of medium expressing plants (3) at 510 nm.

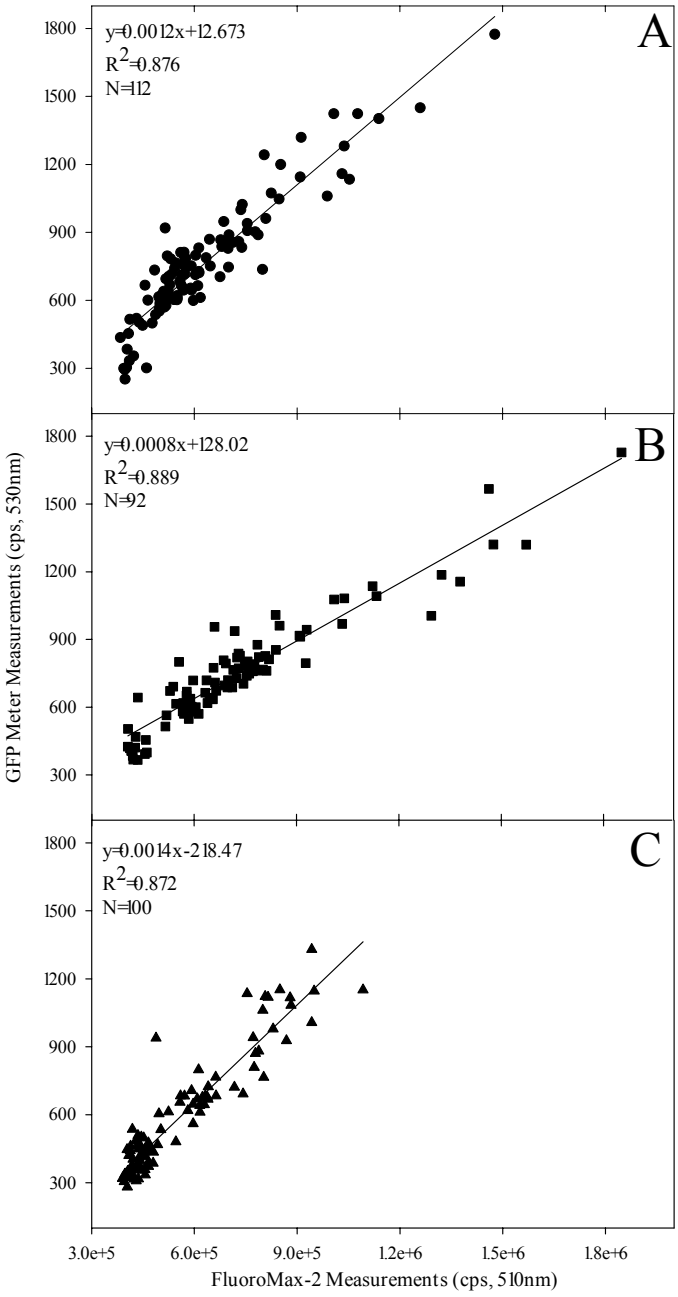

Figure 3. Comparisons of spectrofluorometer (FluoroMax-2) standardized measurements at the 510 nm wavelength and GFP Meter measurements at the 530 nm wavelength. Panel A, GFP measurements of canola grown in an environmental control chamber; panel B, GFP measurements of GFP tobacco grown in a greenhouse; and panel C, measurements of GFP tobacco grown under field conditions (Reidsville, NC). All measurements were recorded on intact or collected leaf tissues, and simple linear regression was performed to compare the measurements of both instruments.

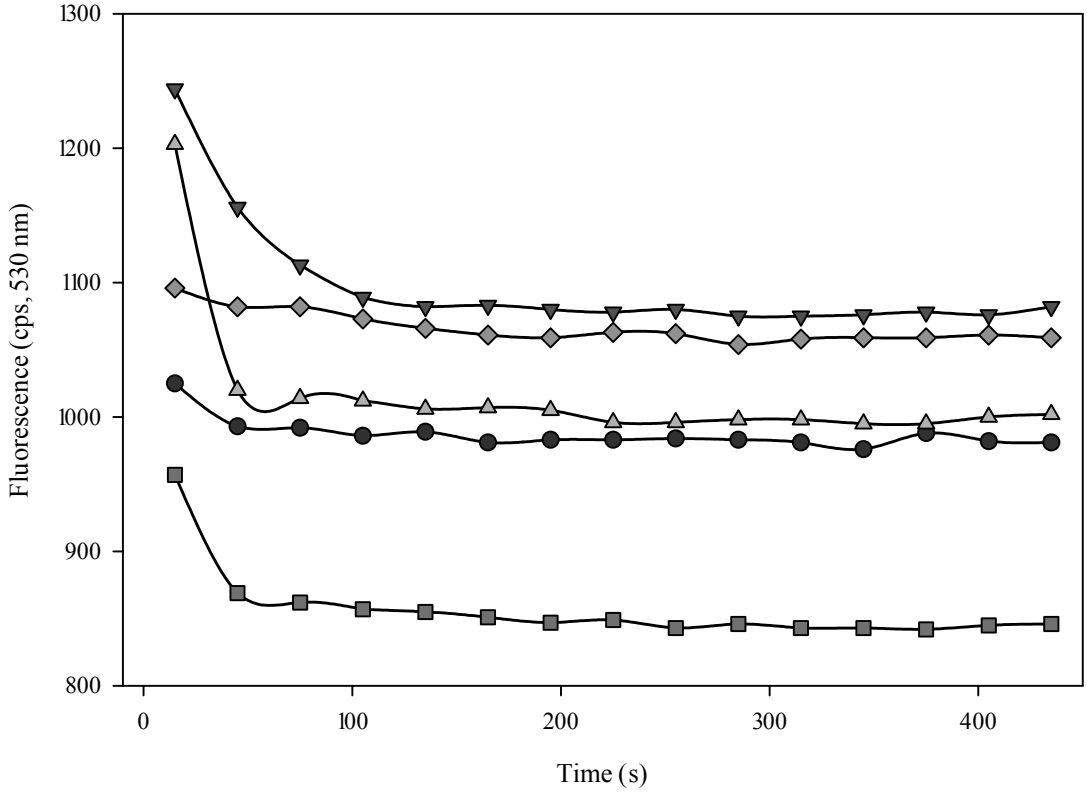

Figure 4. Time course studies using the GFP Meter. Each line represents an individual canola plant. The GFP Meter leaf clip was placed at the same location on a single intact leaf, and GFP fluorescence measurements (530 nm wavelength) were recorded every 13 seconds.

Chapter 4: Spatial and temporal patterns of green fluorescent protein (GFP) fluorescence during plant development in transgenic canola, *Brassica napus* L.

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Abstract

Green fluorescent protein (GFP) has been proposed as a potential marker for the monitoring of transgenic plants and quantifying recombinant protein levels under field conditions. This report characterizes spatial and temporal patterns of GFP fluorescence in transgenic canola (*Brassica napus*, L., cv. Westar) leaf tissue during plant development and the predictive value of GFP fluorescence for the concentration of a genetically linked *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin protein. Under two experimental conditions (environmental chambers and an agronomic field), GFP fluorescence was shown to be variable at each leaf position over time and among different leaves on the same plant. A leaf had its highest GFP fluorescence after emergence, and subsequently, its fluorescence intensity decreased over time. Younger leaves were significantly more fluorescent than older leaves on the same plant. GFP fluorescence intensity was directly correlated with the concentration of soluble protein per unit wet mass ($R^2 = 0.967$ and $R^2 = 0.83$, in

environmental chambers and field conditions, respectively). GFP fluorescence showed no significant correlation to leaf area, wet or dry mass, wet mass per unit area, and relative chlorophyll concentration. At the last harvest date under both laboratory and field conditions, GFP fluorescence was correlated with Bt concentration ($R^2 = 0.60$ and $R^2 = 0.85$, respectively). From these experiments, GFP fluorescence was shown to be a variable characteristic over the period of vegetative development. Fluorescence was correlated with the reduction of soluble protein as leaves age, and that variable fluorescence was indicative of the concentration of a genetically linked recombinant protein.

Key words: green fluorescent protein, Bt, soluble protein concentration, transgene monitoring

Introduction

Monitoring transgenic plants under field conditions will become increasingly important as various genetically modified (GM) crops are implemented in large-scale agricultural applications. The management of escaped transgenic plants in volunteer populations, the presence of unintended transgenes in certified seed lots, and transgenic hybrid populations are fundamental issues yet to be resolved for the sustained use of the products of biotechnology. Monitoring transgenic canola (*Brassica napus*) is especially pertinent, due to significant rates of seed loss during harvesting (Thomas et al. 1991), the abundance of canola volunteers in subsequent years (up to 5.4 plants per m^2, after postemergence herbicide application)(Légère et al. 2001), and the relatively long period of volunteer persistence (5 years)(Simard et al. 2002). Unintended (adventitious) transgene

presence in certified seed lots is also a concern, and Beckie et al. (2003) demonstrated that mixed seed lots could produce individuals that are resistant to multiple herbicides under field conditions. For these reasons, canola is a likely candidate for a transgene monitoring system.

For a monitoring system to be effective, the marker technology should be accurate with few false positives and negatives, detectable throughout the life cycle of the plant, and able to inform on the status of genetically linked transgenes of interest. Green fluorescent protein (GFP) has been proposed as a whole-plant marker for field-level applications (Stewart 1996). The GFP gene was first cloned from jellyfish (*Aequorea victoria*) in 1992, and has since been modified for specific applications and transformed into many different organisms (Chalfie et al. 1992, Prasher et al. 1992, Siemering et al. 1996, Chiu et al. 1996). GFP monitoring has the potential to track transgenes under large spatial scales utilizing visual or instrumental detection of the characteristic green fluorescence of transgenic material. The *mGFP5er* variant gene has been shown to be a feasible transgene monitor in plants under field conditions (Harper et al. 1999). This gene was field tested in tobacco (*Nicotiana tabacum*), and the plants synthesized the protein and remained fluorescent throughout the growing season (Harper et al. 1999). GFP has also been shown to be a feasible qualitative marker for the presence of a linked a synthetic *Bacillus thuringiensis* (Bt) *cry1Ac* endotoxin gene transgene (Harper et al. 1999, Halfhill et al. 2001). With these beneficial characteristics, the next step in the development of a GFP monitoring system is to better describe the system in canola and resolve weaknesses that could limit the utility of the monitoring system.

The utility of a GFP detection system under field conditions could be constrained by differential fluorescence during the plant life cycle. If GFP fluorescence is inconsistent or

absent during specific periods of plant development, the utility of a GFP detection system could be limited. Expression of a GFP transgene regulated by the cauliflower mosaic virus 35S promoter in transgenic canola (*Brassica napus*, L.) has been shown to exhibit inconsistent fluorescence in leaf tissue during the plant life cycle (Halfhill et al. 2001). Tissue-specific differential expression of transgenes regulated by the 35S promoter has been demonstrated in other studies (Odell et al. 1985, Benfey and Chua 1990, Blumenthal et al. 1999, Harper and Stewart 2000). However, it appears to be important to characterize spatial and temporal patterns of GFP fluorescence in canola as suggested by anecdotal evidence (Halfhill et al. 2001). This changing pattern of fluorescence within canola leaves must be understood in order to advance the potential use of a GFP monitoring system.

Two types of experiments were performed to determine the pattern of GFP fluorescence during leaf development in GFP transgenic canola, to describe the cause for variable GFP fluorescence intensities, and to determine the predictability of GFP fluorescence for the concentration of a genetically linked Bt gene. The first experiment was performed in environmental chambers where the growth parameters of the plants could be accurately controlled and quantified. The second experiment was a field-level test to quantify GFP fluorescence in plants under agronomic conditions. In both experiments, GFP fluorescence was tested for correlations against several plant growth characteristics (leaf area, wet and dry mass, wet mass per unit area, chlorophyll content, and soluble protein per unit wet mass) and against concentrations of a linked Bt gene to evaluate the potential utility of a GFP monitoring system.

Materials and methods

Environmental chamber experiment

The environmental chamber experiment was performed in the Southeastern Plant Environmental Laboratory at North Carolina State University (Downs and Thomas 1991). The chambers were programmed for an 8 h photoperiod utilizing high-pressure sodium lamps, and day/night temperatures were maintained at 22/18^{o} C. Three homozygous T_4 GFP canola (*Brassica napus* cv. Westar) events were utilized in this study, GT1 containing GFP (*Aequorea victoria*, *mGFP5er* variant, Haseloff et al. 1997) and Bt (*Bacillus thuringiensis*, synthetic Bt *crylAc*) transgenes, GFP1 and GFP2 containing only the GFP gene (Halfhill et al. 2001), and non-transgenic canola (Westar). A transgenic event was defined as the progeny of a single, independently transformed plant recovered from tissue culture. Four seeds from each transgenic event were sown in a standard greenhouse soil mix in 0.6-l pots (11 x 11 cm)(3 events grown concurrently, 48 pots total). Five non-transgenic plants were maintained as controls. The pots were fertilized daily with a nutrient solution containing 7.6 mM N, 0.3 mM P, 2.8 mM K, 1.4 mM Ca, 0.5mM Mg, and micronutrients in ¼ strength Hoagland's solution. After germination, emerging seedlings were allowed to establish for 1 week, and were culled to one plant per pot.

Four plants per transgenic event (3 events grown concurrently, 12 plants total) were randomly harvested among all the plants in the environmental chambers at eight-day intervals beginning on the 18th day post germination (4 harvests total). At each harvest, leaf area for each leaf position was recorded with a Li-Cor 3100 Leaf Area Meter (Li-Cor Instruments, Lincoln, NE) for all measurable leaves (those greater than 5.0 cm^2). Wet mass for each leaf was recorded. GFP fluorescence and relative chlorophyll concentration were

recorded with the GFP Meter (Opti-Sciences, Inc., Tyngsboro, MA). The GFP Meter was a self-contained, field portable fluorescence detection and data-logging instrument (Millwood et al. 2003). For this study, a 465 nm filter with a bandwidth of 35 nm was used for GFP excitation. To quantify GFP emission, channel 1 (GFP channel) used a 530 nm filter with a bandwidth of 35 nm for emission and channel 2 (chlorophyll channel) used a 680 nm filter with a bandwidth of 35 nm for emission. For each leaf, two GFP measurements were recorded from the underside of the leaf, slightly off the midvein. On the last harvest date (42 d), GFP fluorescence was recorded for each leaf position for the five non-transgenic canola (Westar) plants. For the transgenic samples at each harvest date, two 1-cm diameter leaf punches were collected from fresh leaves from the same position as the GFP measurements from each leaf with a 1.5 ml microcentrifuge tube. Each individual leaf was placed a into paper bag, and dried in a convection oven at 60^{o} C for 72 h. Dry weight of each leaf was recorded.

Soluble protein was extracted from the leaf punches, and Bradford Analysis (Bio-Rad Laboratories, Hercules, CA) was used to quantify soluble protein concentration. The mass of each punch was recorded, and the sample was homogenized with 0.5 mm glass beads in a mechanical amalgamator (Silamat S5, Ivoclar Vivadent Clinical, Austria). After disruption of the leaf tissue, 400 µl of 0.1 N NaOH was added to each sample, and the sample was incubated in ice for 30 minutes. After incubation, 80 µl of 1M Tris-HCl pH 4.5 was added to each sample for neutralization. The sample was centrifuged for 7 min at 7000 rpm, and the supernatant containing soluble protein was recovered. Bradford Analysis was used to determine soluble protein concentrations in 96 well plates (EL 800 Universal Microplate Reader with the KC Junior software package, Bio-Tek Intruments, Inc., Winooski, VT). Bt

protein concentration was determined by the use of a Bt ELISA (Cry1Ab/Cry1Ac Plate Kit, Catalog No. AP 003, Envirologix, Inc., Portland, ME).

At each harvest date, ANOVA was utilized for evaluating differences in GFP fluorescence, and Fisher's PLSD was used to determine where significant differences occurred between leaf positions and collection dates (StatView 5.0 for Windows, 1992-1998, SAS Institute Inc., Cary, NC). Regression analysis was performed for GFP fluorescence by leaf area, leaf age, leaf wet and dry mass, wet mass per leaf area, chlorophyll concentration, Bt concentration per unit wet mass, and concentration of soluble protein per unit wet mass (StatView 5.0 for Windows).

Field experiment

A GFP *Brassica napus* field experiment was conducted at the Central Crops Research Station, Clayton, NC, USA (35^{o} 39'N 78^{o} 27'W) in the spring of 2001. Ten events of GFP *B. napus* (GT 1-5, GT 8-9, and GFP 1-3) were hand planted in ten 2 × 48 m plots, one event per plot (March 27, 2001). The planting rate was 100 seeds per m^2 for a final stand density of 50 plants per m^2 based on 50% predicted germination frequency. Beginning twenty-four days post germination, five plants per event were harvested from the field at seven-day intervals (50 plants per harvest, six harvests total), and GFP fluorescence per leaf position was recorded with a Fluoromax-2 spectrophotometer. A Fluoromax-2 spectrophotometer (Jobin Yvon & Glen Spectra, Edison, NJ, USA) with DataMax and GRAMS/386 software (Galactic Industries Corporation, Salem, NH, USA) was used to quantify GFP fluorescence in the leaf tissue. Each leaf 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*.

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 (Millwood et al. 2003). Relative chlorophyll concentrations were estimated at the same position as GFP fluorescence using a Minolta SPAD-502 Chlorophyll Meter (Spectrum Technologies, Plainfield, IL). Two 1-cm diameter leaf punches were collected from fresh leaves from the same position as the GFP measurements from each leaf with a 1.5 ml microcentrifuge tube. Soluble protein was extracted, and soluble protein and Bt concentration per unit mass were quantified as above. At each harvest date, ANOVA was utilized for evaluating differences in GFP fluorescence, and Fisher's PLSD was used to determine where significant differences occurred between leaf positions and collection dates (StatView 5.0 for Windows). GFP fluorescence was correlated by regression to relative chlorophyll concentration, Bt concentration per unit wet mass, and concentration of soluble protein per unit wet mass (StatView 5.0 for Windows).

Results

Patterns of GFP fluorescence

Under both sets of experimental conditions, GFP fluorescence (530 nm) displayed two specific patterns. First, GFP fluorescence decreased at each leaf position over time, and second, different leaf positions on the same plant demonstrated a variable pattern of GFP fluorescence with younger leaves fluorescing significantly more than older leaves (Figure 1). In the environmental chamber experiment, canola event GT 1 plants at the four-leaf stage exhibited similar GFP fluorescence among all leaf positions (18 d)(range 975.9 ± 16.2 to

1067 ± 20.2, average counts per second (cps) ± standard error). At the next harvest date (26 d), GFP fluorescence decreased at leaf position 1 and 2 by ca. 22%, and the youngest leaves at leaf positions 3-6 showed significantly higher GFP fluorescence compared with older leaves (range 1026.0 ± 44.1 to 1174.5 ± 24.1). These trends continued at the 34 d harvest, with GFP fluorescence decreasing at the older leaf positions (1-4) by 22% - 37% from the previous harvest date, and the youngest leaves (positions 5-8) had higher GFP fluorescence compared to the older leaves (positions 1-4). At the 34 d harvest, the older leaves demonstrated similar fluorescence intensity as non-transgenic canola plants. Non-transgenic leaves had an average fluorescence at 530 nm of 551.9 ± 17.4 with no significant differences among leaf positions (ANOVA, $P > 0.05$)(data not shown). At the last harvest date (42 d), all older leaf positions (1-8) exhibited the characteristic decrease in fluorescence with leaf positions 1-3 similar to non-transgenic canola leaves, and the youngest leaves (positions 9 and 10) had the highest average GFP fluorescence. These patterns were similar in all transgenic events in both experimental conditions (data not shown).

GFP fluorescence was correlated with soluble protein

Once the pattern of GFP fluorescence was described, we examined other factors that might be associated with decreasing GFP fluorescence over time. The relationship between GFP fluorescence and leaf area, wet and dry mass, wet mass per unit area, and relative chlorophyll concentration at each harvest date was analyzed by simple-linear regression, and no significant effects were found ($P > 0.05$)(data not shown). In contrast, GFP fluorescence was significantly positively associated with soluble protein per unit wet mass (μg/μl/g tissue) in leaves of all ages (Figures 2 and 3). In environmental chambers and under field conditions

on the last harvest date (42 d and 59 d, respectively), GFP fluorescence was directly proportional to the soluble protein per unit wet mass (environmental chamber GT1 $R^2 = .967$; field experiment over 8 combined canola events $R^2 = 0.83$). In the environmental chamber experiment, GT1 had this positive association over all plant harvest dates where GFP fluorescence varied over leaf positions (harvests 26 d – 42 d)(Figure 2). At the 42 d harvest, canola events GFP 1 and GFP 2 exhibited significant correlations between these indices (P = 0.0198 and 0.0483, respectively), although the R^2 values for these regression lines were less robust ($R^2 = 0.563$ and 0.449, respectively)(data not shown).

Predictive value of GFP fluorescence for the concentration of a genetically linked protein

For all GFP/Bt events in both experiments, GFP fluorescence was significantly associated with Bt concentration (Figure 4). In the environmental chamber experiment, GFP fluorescence was associated with Bt concentration (μg Bt/g tissue) at the last harvest date (48 d, $R^2 = 0.604$)(data not shown). Under field conditions when the eight GT events were combined, the GFP fluorescence intensity was strongly associated with Bt concentration at the last harvest date (59 d, $R^2 = 0.8532$)(Figure 4). In this case, the concentration of both genetically linked proteins exhibited the same pattern of variation with the highest concentrations in youngest leaves. Therefore, GFP fluorescence could be used as an indicator of the level expression of another recombinant protein that was linked to GFP genetically.

Discussion

The results of this research indicate that GFP fluorescence in GFP transgenic plants followed two specific patterns. First, fluorescence intensity at 530 nm decreased at each leaf position over time. Second, fluorescence differed among leaf positions on the same plant with the highest fluorescence observed in young leaves. GFP fluorescence intensity was highest in young leaves up to two weeks after emergence, then the fluorescence intensity decreased over time to levels observed in non-transgenic controls as leaves aged. Therefore, when a plant has a large number leaves at various ages, a wide spectrum of GFP fluorescence can be detected, ranging from the highest level observed in that transgenic event at and near the apical meristem to those similar to non-transgenic levels in old leaves. In leaf tissues, where the cauliflower mosaic virus 35S promoter produces a consistent percentage of recombinant protein per unit total soluble protein (Blumenthal et al. 1999, Harper and Stewart 2000), GFP fluorescence consistently varied with the concentration of soluble protein in a mass of fresh leaf tissue. This research has shown that soluble protein per unit leaf mass changes during leaf development, and the GFP phenotype is correlated with this phenomenon.

The two patterns of GFP fluorescence described in these experiments are consistent with the pattern of sequential senescence of leaves described in the plant physiology literature for the last 40 years (Woolhouse 1967, Friedrich and Huffaker 1980, Huffaker 1982). Sequential senescence describes the pattern of canopy development in which a leaf initially emerges and soluble protein content increases during leaf elongation. The soluble protein concentration reaches an upper threshold, and then decreases with time until the leaf is eventually shed (Woolhouse 1967). The concentration of single proteins within the total

soluble protein have been described during this process, and Friedrich and Huffaker (1980) showed that ribulose-1.5-bisphosphate carboxylase (RuBPCase) concentration was initially at high levels in young leaves, and decreased over time in a correlated fashion to soluble protein in primary barley (*Hordeum vulgare*) leaves. GFP fluorescence is consistent with the process of sequential senescence, with young, expanding leaves fluorescing at high levels as soluble protein concentration increases, and then decreasing over time as the soluble protein decreases until the leaves would be eventually shed. GFP fluorescence in transgenic plants is closely tied to the pattern of soluble protein concentrations in leaves over time.

A monitoring system that utilizes GFP fluorescence must take into account the changing patterns of fluorescence to provide an accurate assessment of transgene expression. The GFP phenotype was variable during canola leaf development and correlated with the concentration of soluble protein in leaf tissues. A GFP monitoring system therefore can generate false negatives if one solely assays older leaves having lower concentrations of soluble proteins. When the 35S promoter regulates GFP, a GFP monitoring system may be confounded for qualitative assessments of transgenic status if only older leaves are scored for GFP fluorescence. This potential problem may be overcome if qualitative assessments are taken from young leaves near the apical meristem.

GFP fluorescence can be used to quantify the concentration of a genetically linked protein of interest. This was supported in this study by GFP fluorescence accurately predicting the Bt concentration in leaf tissues. It is conceivable that the value of GFP fluorescence as a predictor of expression of a linked gene could be decreased if the genes become unlinked through recombination or if transgene silencing occurs. The variable patterns of GFP fluorescence in developing leaves was an accurate predictor of soluble

protein concentration, and may prove to be a significant tool for the determination of the concentration of economically important recombinant proteins for other applications or for simply monitoring general plant health and stress.

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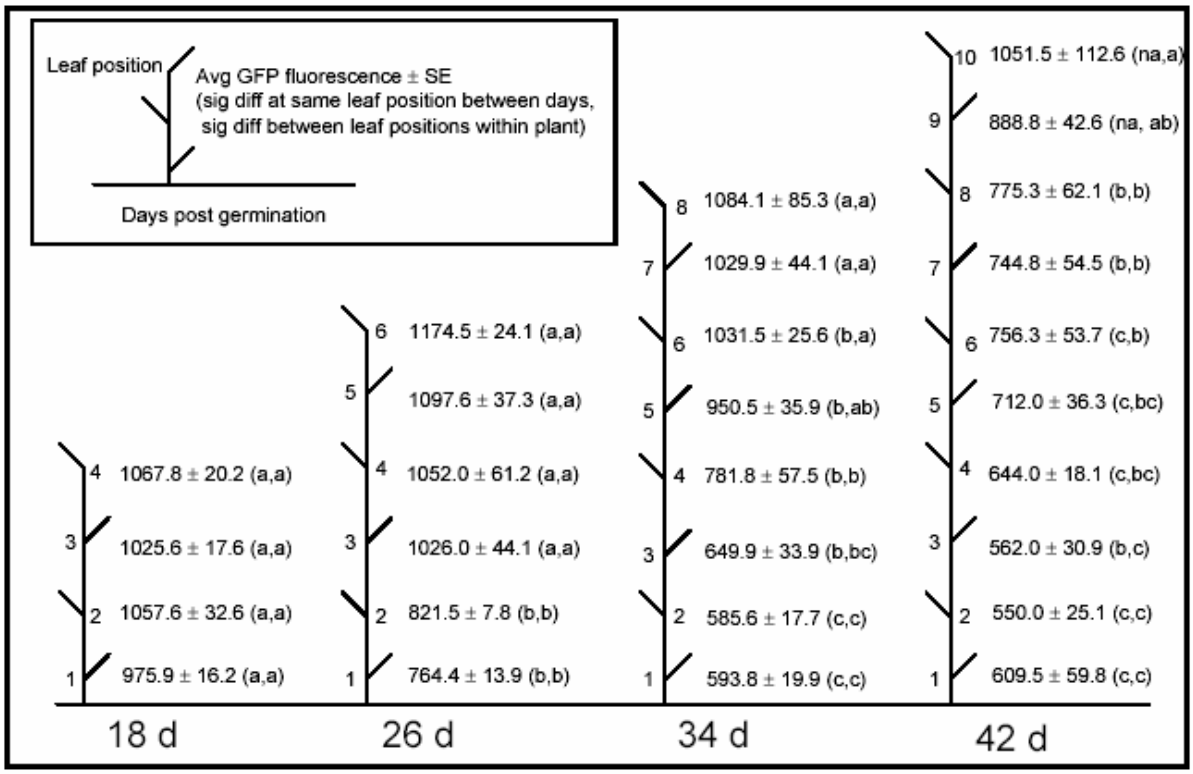

Figure 1. GFP fluorescence (530 nm) intensity at each leaf position of GT1 canola grown in environmental chambers. GFP fluorescence was quantified with the GFP Meter (units in counts per seconds, 530 nm). Non-transgenic canola demonstrated an average 530 nm fluorescence of 551.9 ± 17.4 (± SE). Different letters represent significant differences (Fisher's PLSD, $P < 0.05$).

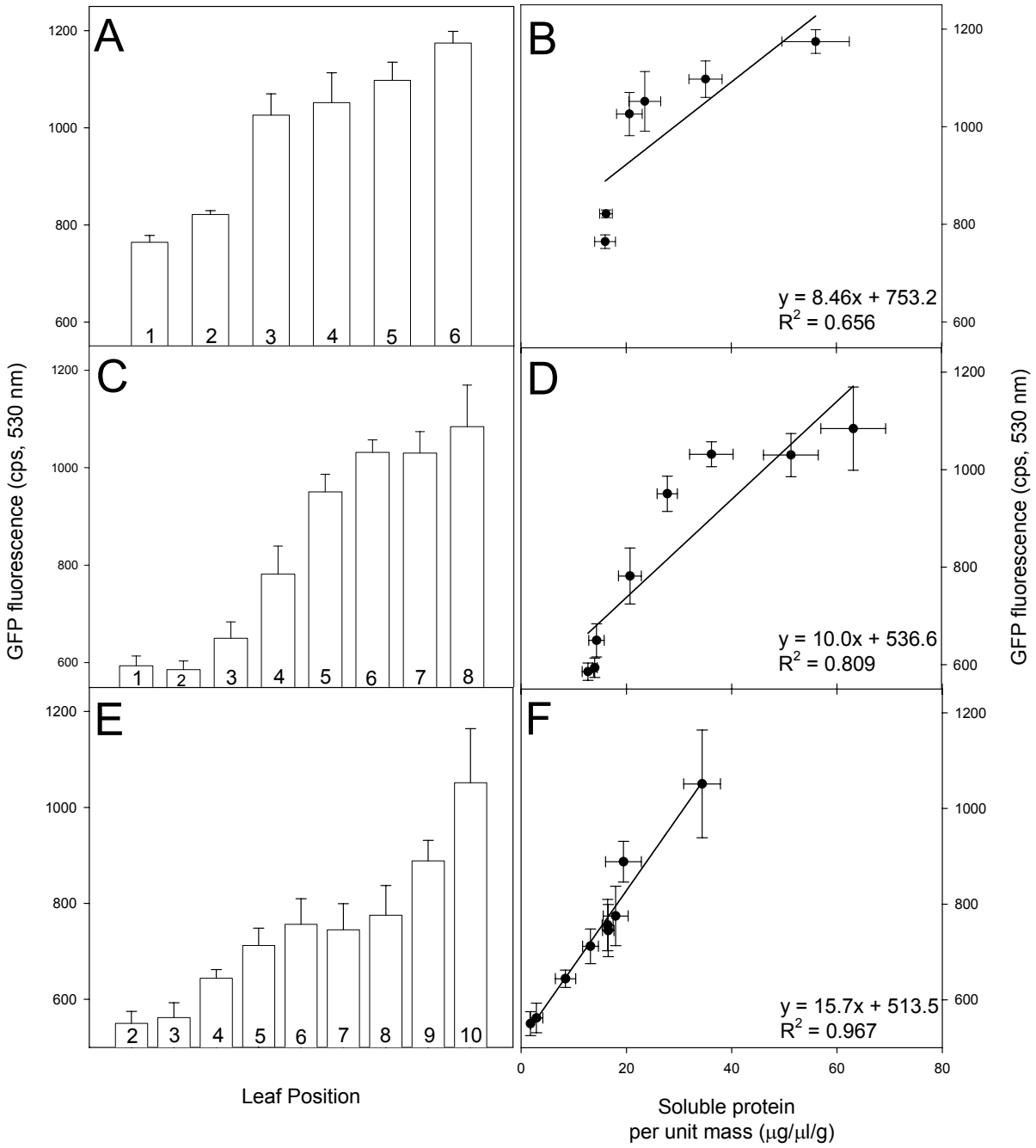

Figure 2. GFP fluorescence was associated with the concentration of soluble protein per leaf wet mass. Three harvest dates from canola event GT1 grown in environmental chambers are represented (Panels A and B, 26 d; panels C and D, 34 d, panels E and F, 42 d).

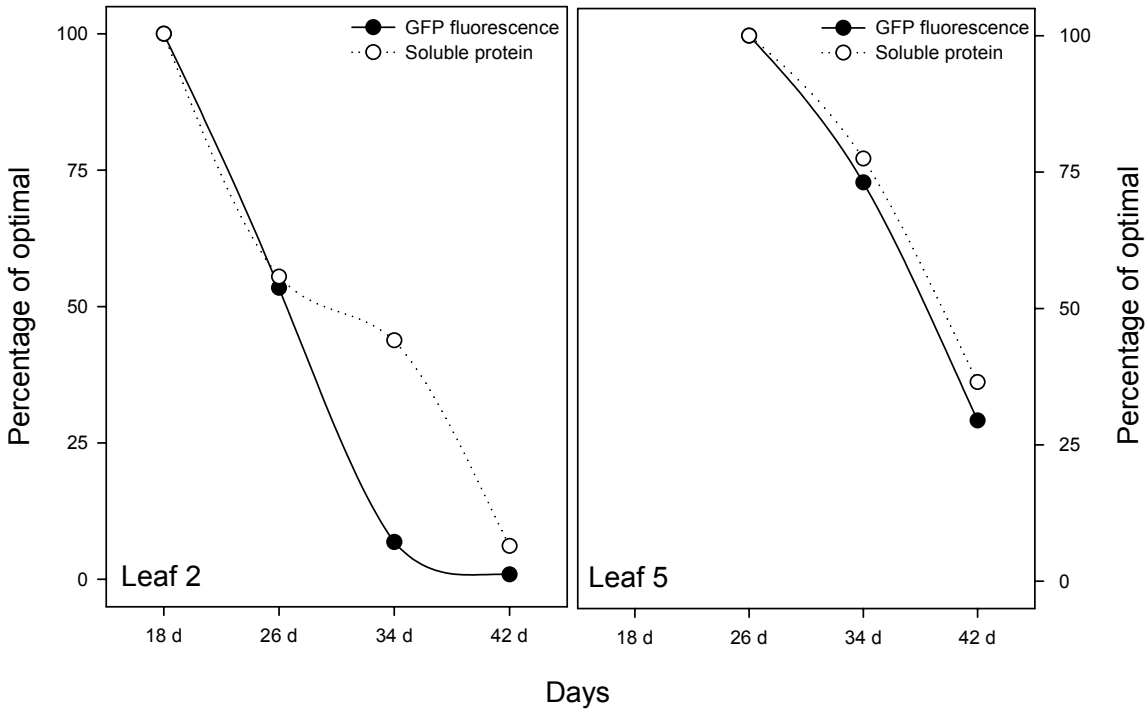

Figure 3. Percentage of optimal soluble protein and GFP fluorescence at leaf positions 2 and 5. Optimal values were the highest average value for each measurement at each harvest date. The samples were from canola event GT1 grown in environmental chambers. GFP fluorescence values were calculated by subtracting the wild-type canola (Westar) fluorescence at 530 nm. The decrease of the concentration of soluble protein and GFP fluorescence was consistent with the process of sequential senescence.

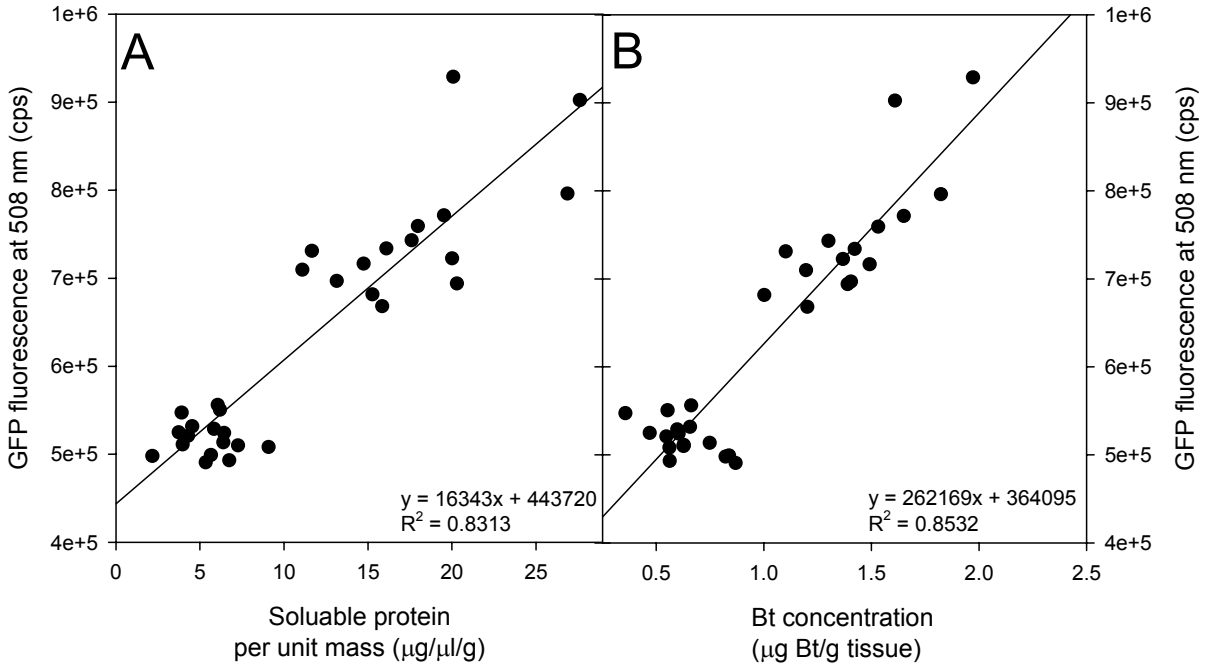

Figure 4. GFP fluorescence was associated with soluble protein per unit leaf mass and Bt concentration under field conditions 59 days post germination. Data points represent leaf positions 3, 5, 9, and 11 from 8 combined transgenic GFP/Bt canola events (GT 1-6, 8, and 9).

Chapter 5: Additive transgene expression and genetic introgression in multiple green fluorescent protein transgenic crop x weed hybrid generations

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Abstract

The level of transgene expression in crop x 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. 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 (*Brassica napus*, cv. Westar) and birdseed rape (*B. 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 domestics* 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 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.

Key words: transgenic canola, *Brassica rapa*, green fluorescent protein, AFLPs, gene flow

Introduction

Movement of transgenes from transgenic canola (*Brassica napus* L., AACC, 2*n* = 38) into different canola varieties and weedy relatives is a potential and realistic risk associated with 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 (*B. rapa* L., AA, 2*n* = 20) and distant (*Raphanus raphanistrum* L., RrRr, 2*n* = 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 weeds exhibiting transgenic phenotypes are likely to occur in agricultural fields where crops may cross-pollinate with weedy species (Metz et al. 1997, Halfhill et al. 2001, Halfhill et al. 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 homozygous versus hemizygous status (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 x *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 nondestructive, *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 (*Brassica 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) and three GFP (GFP 1-3) events 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 x canola event produced the F_1 hybrid generation, and *B. rapa* x 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 hybrids generations (*Brassica rapa* CA, 2974, 2975 x GT 1-9) and nine GFP hybrids generations (*B. rapa* CA, 2974, 2975 x GFP 1-3) were hand-crossed with the respective parental accession of *B. rapa* (CA from Irvine, California, USA, courtesy of Art Weiss; 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 (CA x GT1, 2974 x GT1, and 2974 x 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 (*Brassica 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 x 4 in pots and grown in controlled-environment growth chambers (Percival Scientific, Perry, Iowa, USA) at $22^{o}/18^{o}$C under a 12/12 hour 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 & Glen Spectra, Edison, NJ, USA) with DataMax and GRAMS/386 software (Galactic Industries Corporation, Salem, NH, 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* (CA and 2974), and successive hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk) from three crossing lines (CA x GT1, 2974 x GT1, and 2974 x GT8) were germinated on filter paper and selected for GFP fluorescence. Ten plants were analyzed for each generation, except 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 420-600 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, *in press*). 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 propidium iodide 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* (CA, 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* x GT *B. napus* (CA x GT1, 2974 x GT1, and 2974 x GT8) were analyzed. Six plants per generation 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 2X CTAB procedure (Doyle & 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 *Eco*R1 and 1.25 U *Mse*1 (Invitrogen) in a 5X reaction buffer (50 mM Tris-HCl pH 7.5, 50 mM MgAc, 250 mM KAc) in a reaction volume of 12.5 µl at 37ºC for 2.5 h, and the restriction enzymes were heat inactivated at 65ºC for 10 min. The digested DNA samples were ligated to adaptors in a ligation solution containing 0.5 U of T4 DNA Ligase, 5X ligation buffer (Invitrogen), 0.4 mM ATP, 1 pMol *EcoR1* adapter and 10 pMol *Mse*1 adapter (Cortec) in a reaction volume of 25.0 µl at 22ºC for 2 h (Table 1). The adapter ligated DNA was diluted 10-fold with 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 dNTP's, 1.5 mM $MgCl_2$, 0.5 U Taq (Roche), 10X 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 dNTP's, 1.5 mM $MgCl_2$, 0.5 U Taq (Roche), 10X 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 were taken from the protocol of 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 hr. Infrared gel images were analyzed using a GeneIR (Scanalytics) program (LI-COR). *Brassica 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.

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 (Figures 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).

Ploidy determination

The ploidy of the three BC_2F_2 Bulk generations was indistinguishable from *B. rapa* when analyzed through flow cytometry (10 plants per generation, 30 total) (Fig. 3). When parental canola and *B. rapa* nuclei were mixed, the respective G1 peaks separated by ~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).

AFLP analysis

The five primer pair combinations generated a total of 270 consistently amplified AFLP bands (Fig. 4), 92 of which were *Brassica 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 ± standard deviation). The BC_2F_1 generation continued this general trend, but demonstrated a wide range of crop-specific markers between the crossing lines. The CA x GT1 and 2974 x GT1 BC_2F_1 generations had lower percentages of these markers: 30% ± 7 and 25% ± 3, respectively, in contrast to the 2974 x 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, BC_1F_1, and BC_2F_1) and 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 ± standard deviation 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 x GT1 and 2974 x 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 10 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 toward 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 measurement 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 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. Vain et al. (1999) quantified GFP fluorescence in transgenic *A. thaliana* lines 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 & Glen Spectra, Edison, NJ), 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, MA) 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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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'

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

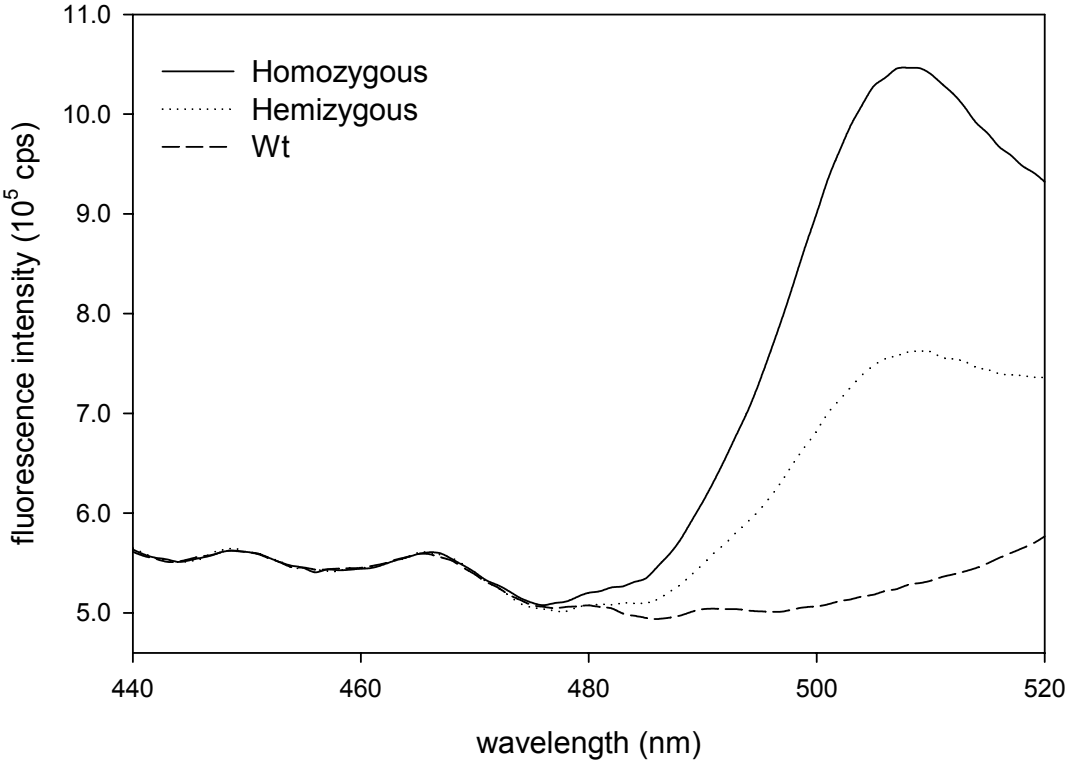

Figure 1. Average scanning fluorescence emission of homozygous versus hemizygous individuals of T_1 GFP2 canola when excited with 385 nm UV light. Non-transformed canola (Westar, Wt) was used as a control. Homozyogous individuals exhibited an average fluorescence at 508 nm of 10.5 ± 0.4 (all units in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

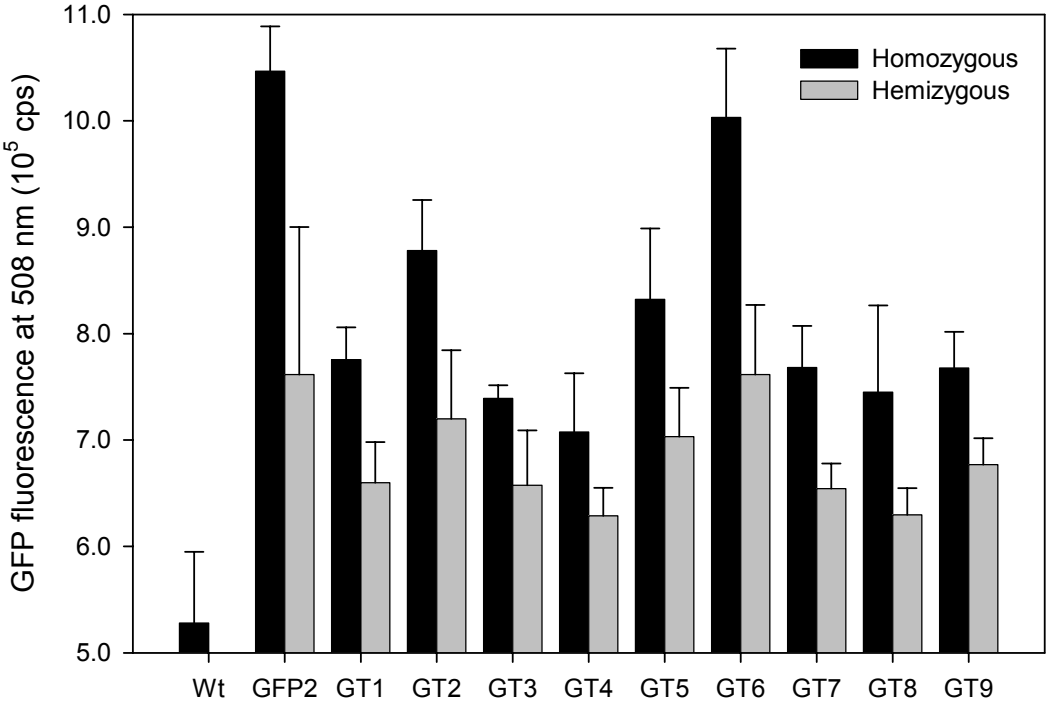

Figure 2. GFP fluorescence at 508 nm for homozygous versus hemizygous T_1 plants within 10 transgenic canola lines when excited with 385 nm UV light. Non-transformed canola (Westar, Wt) was used as a control. Homozygous individuals fluoresced significantly higher at 508 nm compared to hemizygous individuals ($P<0.05$).

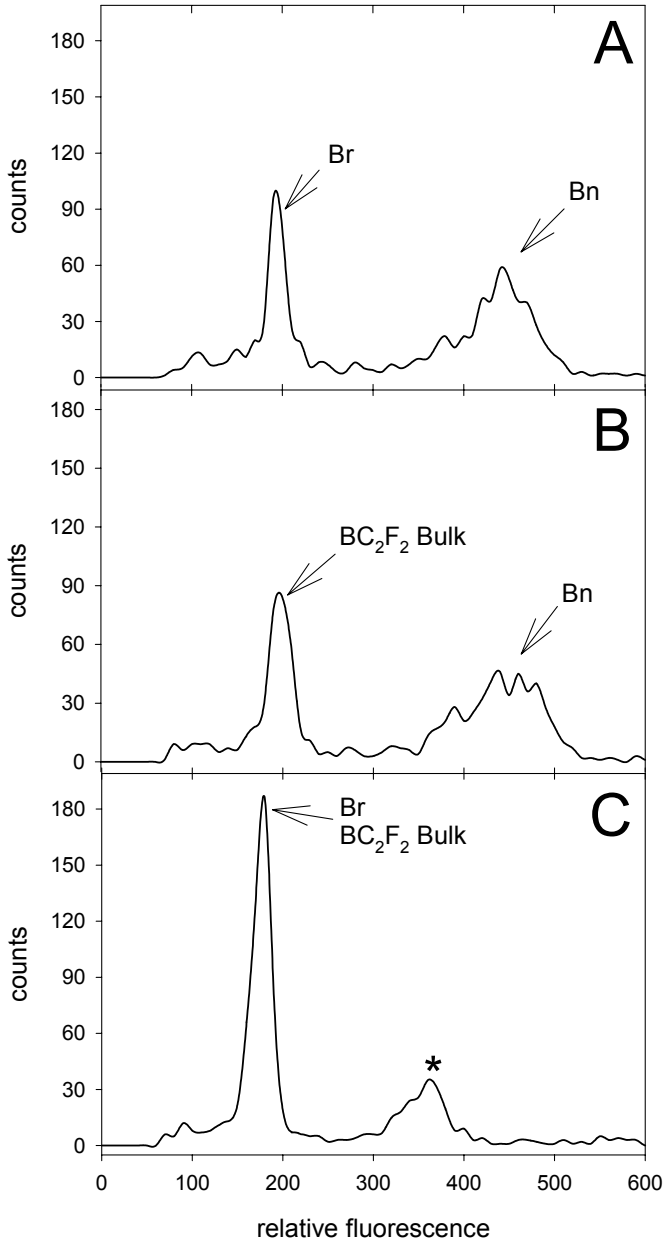

Figure 3. Relative DNA content of canola (*Brassica napus*, Bn), birdseed rape (*B. rapa*, Br), and BC_2F_2 Bulk hybrids as determined by flow cytometry. Panel A, histogram of flow cytometric analysis of mixed samples of parental canola and birdseed rape nuclei. Panel B, histogram of BC_2F_2 Bulk nuclei mixed with canola, and Panel C, histogram of BC_2F_2 Bulk nuclei mixed with birdseed rape. The arrows in each panel demark 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.

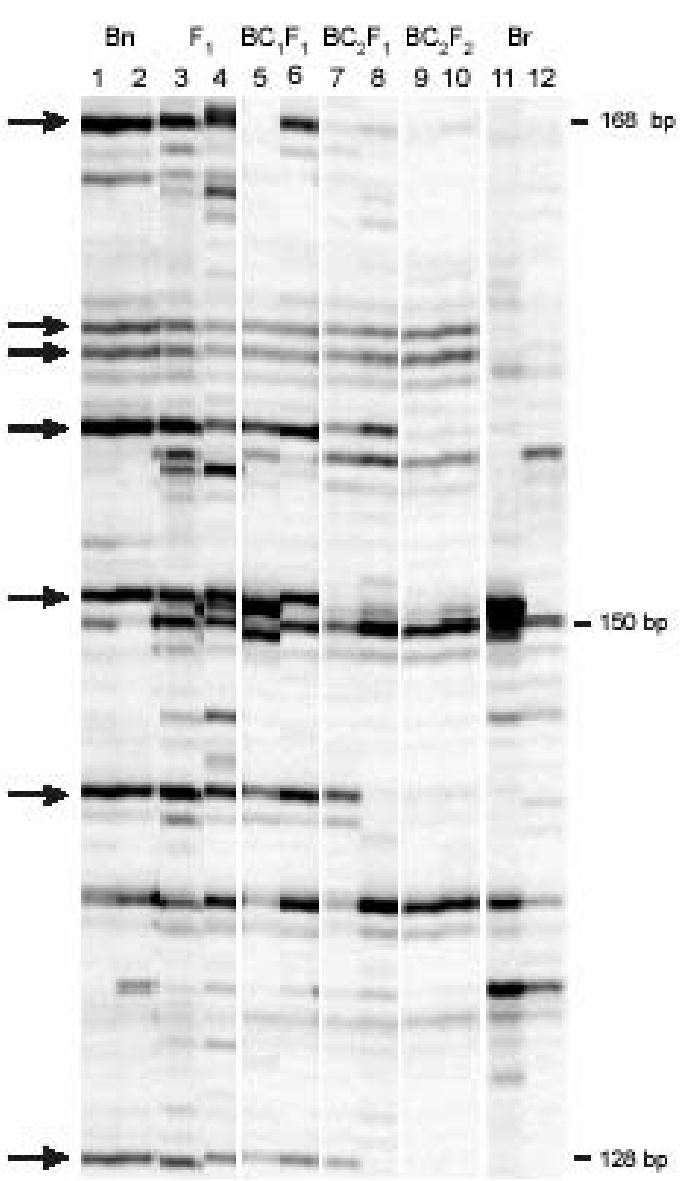

Figure 4. AFLP analysis of canola (*Brassica napus,* GT1; Bn, lanes 1-2), hybrid generations (CA x GT1: F_1, 3-4; BC_1F_1, 5-6; BC_2F_1, 7-8, BC_2F_2 Bulk, 9-10), and *B. rapa* (CA; Br, 11-12). Arrows represent *B. napus*-specific AFLP markers.

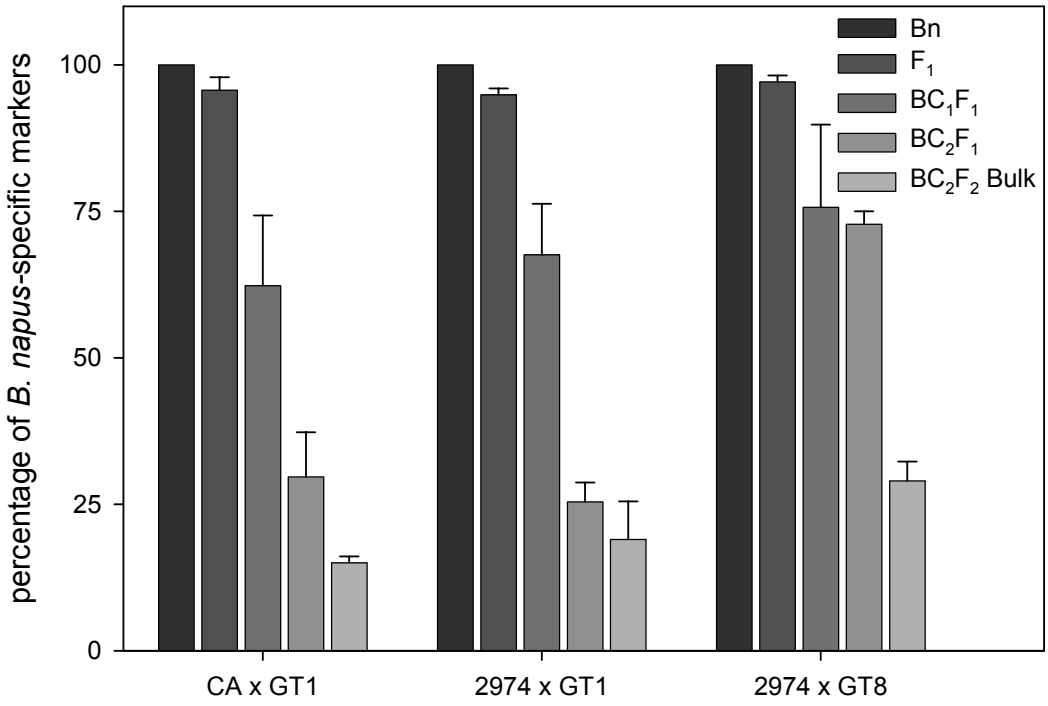

Figure 5. AFLP analysis of canola (*Brassica napus*, Bn) and F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk hybrid generations. AFLP analysis with five specific primer sets yielded 92 *B. napus*-specific markers. Within each crossing line (CA x GT1, 2974 x GT1, 2974 x GT8), percentage of Bn specific markers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

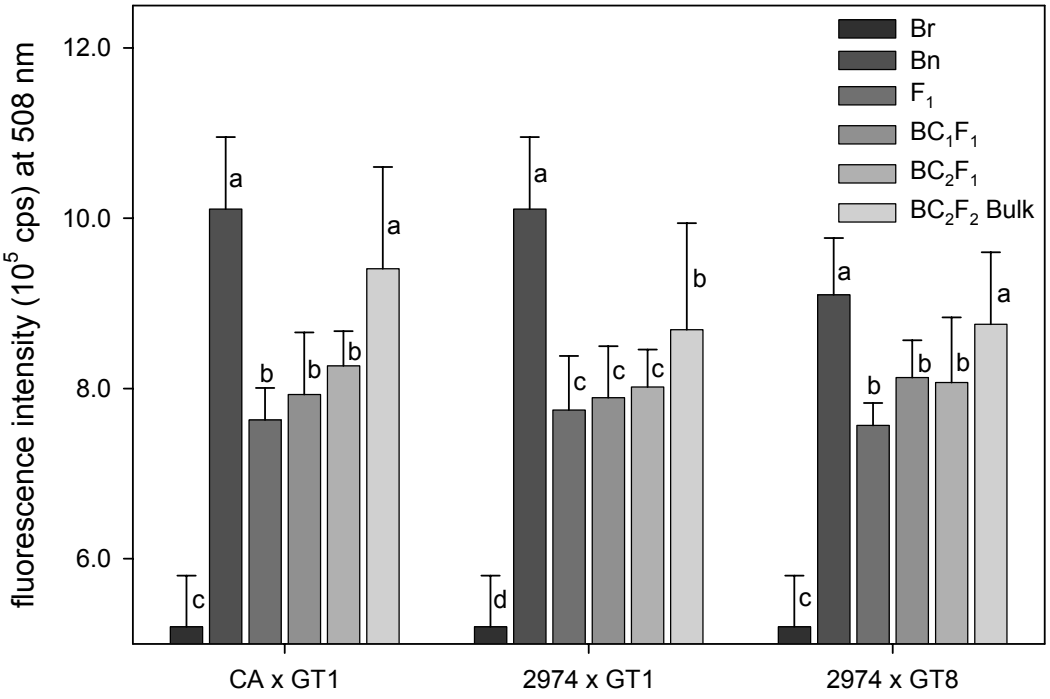

Figure 6. Average GFP fluorescence of parental transgenic canola (Bn) and F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk hybrid generations with *B. rapa* (Br). Leaves were excited with 385 nm UV light and fluorescence intensity was measured at 508 nm (cps). Fluorescence averages (± standard deviation) per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

Chapter 6: Altered vegetative growth and competitiveness associated with gene flow from transgenic *Brassica napus* (canola) to weedy *B. rapa*

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Abstract

Concerns exist that transgenic crop × weed hybrid populations will be more vigorous and competitive with crops compared to parental weed species. Two experiments were performed to evaluate the consequences of introgression of transgenes into hybrid generations on crop production. First, vegetative growth potential and nitrogen use efficiency was analyzed for transgenic hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk) along with transgenic *Brassica napus* (canola) and non-transgenic *B. rapa* under optimal (high N) and sub-optimal (low N) conditions in continuous-flow hydroponic chambers. Under optimal conditions, *B. rapa* exhibited higher rates of growth when compared to canola and transgenic hybrid generations. Under sub-optimal conditions, parental and hybrid generations exhibited similar growth indices. Second, the competitive

ability of transgenic hybrids, non-transgenic hybrids, canola and *B. rapa* when grown with a model crop, wheat (*Triticum aestivum*), was quantified by determining crop reduction under field conditions. Transgenic hybrids (BC_2F_2 Bulk) were the least competitive with wheat when compared to the other *Brassica* competitors (25.5% versus 47.1% crop reduction, respectively). From these experiments, wild *B. rapa* demonstrated the greatest potential for growth. Hybridization and transgene introgression resulted in populations with lower vegetative growth potential and competitive ability. Although backcrossing resulted in transgenic hybrid generations with similar ploidy and morphologic characteristics as *B. rapa*, the vegetative growth and competitive ability of hybrid generations was reduced, and may be due to many factors including the co-introgression of crop-adapted genes that would cause a semi-domestication of the weed.

Key words: transgenic canola, *Brassica rapa*, vegetative growth, green fluorescent protein, hybridization

Introduction

Genetically modified crop varieties have been increasingly grown in commercial agriculture. Concerns exist over the potential for transgene flow to wild relatives and the production of transgenic hybrid populations. A risk with the inadvertent production of transgenic weeds via hybridization with crops is an increase in fitness and weediness. It is conceivable that hybridization and introgression of transgenes may modify the physiological and ecological characteristics of weeds, which could increase competitiveness with cultivated crops.

It has been suggested that gene flow and changes in fitness could be a particular problem with canola (*Brassica napus* L., AACC, 2n=38)(Jorgensen and Anderson 1994, Warwick et al. 1999, Snow 2002). A large percentage of canola varieties used in commercial production are genetically modified. Sexually compatible wild relatives occur commonly near fields in which the crop is grown. Indeed, experimental results have shown that gene flow can occur between species and, based primarily on reproductive indices, that hybrids may be more fit than the *B. rapa* parent (Hauser et al. 1998). Also, in a backcrossing study with herbicide resistant canola, gene flow to *B. rapa* had no negative reproductive costs due to introgression suggesting that transgenes can persist in weed populations (Snow et al. 1999).

The evidence currently available supports the idea that substantial gene flow can occur in agricultural settings. In this series of experiments, we examined growth potential and competitiveness of transgenic hybrid populations produced by crossing transgenic canola and *B. rapa*. The experiments were conducted using four hybrid generations from canola and *B. rapa* crosses that had different genomic compositions ranging from triploid (AAC) to diploid (AA) through progressive backcrossing to *B. rapa* (Halfhill et al. 2002). Seedling vigor and early growth responses may be important vegetative indices to describe how hybrid populations compete in the important period of canopy development. To quantify physiological differences among populations, differential growth responses to optimum (high N) and sub-optimum conditions (low N) in hydroponics were examined for wild *B. rapa*, *B. rapa* × canola hybrid generations, and transgenic canola. In order to assess the risks associated with transgenic hybrids to crop production, competition with a model crop species (wheat, *Triticum aestivum* L.) was analyzed under field conditions. Combined together,

these experiments provided experimental data to evaluate the consequences of gene flow to weeds on crop production.

Materials and methods

Plant breeding

Six plant generations described in Halfhill et al., (2001 and 2003) were utilized in this study: green fluorescent protein (GFP)/*Bacillus thuringiensis* (Bt) transgenic canola (*Brassica napus* L.), birdseed rape (*B. rapa* L.), and transgenic crop × weed hybrid generations (F_1, BC_1F_1, BC_2F_1, BC_2F_2 Bulk)(Figure 1). In summary, one T_2 line of transgenic canola (*B. napus* L. cv Westar, GT1) and a weedy accession of *B. rapa* (2974: Milby, Québec, Canada (45^o 19'N 71^o 49' W), germplasm collection AAFC-ECORC, Ottawa) were hand-crossed to generate a F_1 hybrid generation. Non-transgenic *B. rapa* and homozygous GT1 canola seeds were germinated in soil (4 x 4 in pots), and grown in controlled-environment growth chambers (Percival Scientific, Perry, Iowa, USA) at 22^o/18^{o}C under a 12/12 hour light/dark cycle. All plants were allowed to flower, and hand crossing was performed by pollinating six self-incompatible *B. rapa* plants with pollen from three GT1 plants. All seeds were collected from the *B. rapa* parents, and bulked to produce the F_1 hybrid generation. A subset of the F_1 seeds were germinated on moist filter paper, and fluorescent individuals were 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). Transgenic F_1 hybrids plants were used as pollen donors and backcrossed with the parental *B. rapa* accession in the same fashion as above to produce a BC_1F_1 hybrid generation and subsequently a BC_2F_1 hybrid generation.

The BC_2F_1 plants were intermated to produce BC_2F_2 Bulk populations by placing 27 individuals in a greenhouse enclosure with pollinating insects, houseflies (*Musca domestics* L.). Seeds of the BC_2F_1 generation were germinated in soil. Transgenic BC_2F_1 and non-transgenic individuals were selected for GFP fluorescence by visual assay with a UV light. Transgenic and non-transgenic plants were grown within separate greenhouse enclosures, and houseflies were added as the plants began to flower. Due to the self-incompatibility of the BC_2F_1 plants, insect pollinators were required in order to generate a large number of random pollination events. Seeds were collected from all individuals within each enclosure and bulked together to form BC_2F_2 Bulk generations.

Germination conditions for hydroponics

Three experimental conditions (optimal, sub-optimal, and a temperature range at optimal conditions) in hydroponics were used to determine differences in growth response among the generations. Seeds were germinated with the same methodology for all three experimental conditions in rolls of moist paper in 4-l beakers in 200 ml of 0.1 mM $CaSO_4$ solution, and were placed in a germination chamber at 26^o C for 72 h in the dark. Transgenic individuals were selected for the GFP phenotype on the germination paper by visual assay with a UV spotlight. Seedlings of uniform size (4-5 cm) were selected, and were transferred to continuous-flow hydroponic systems in environmental chambers in the North Carolina State University Phytotron.

Optimal and sub-optimal growth experiments

Optimal (high N) and sub-optimal (low N) experiments were conducted in four, 200-l hydroponic systems in the Southeastern Plant Environmental Laboratory at North Carolina State University (Downs and Thomas 1991). The environmental chamber was maintained at full intensity lighting with an 8 hr day length at 18^{o}/22^{o} C. Each hydroponic system initially contained 40 individuals of the same generation. The total nutrient solution was based on a modified Hoagland's solution (200 μM KH_2PO_4, 300 μM $MgSO_4$, 800 μM $CaSO_4$, 35.8 μM Fe as Fe-Sequestrene, 19 μM H_3BO_3, 3.7 μM $MnCl_2H_2O$, 317 nM $ZnSO_4$, 132 nM $CuSO_4$, and 50 nM 85% H_2MoO_4) with two N levels; high N (maintained at 1000 μM KNO_3) and low N (added daily to a level of 20 μM KNO_3). The nutrient solution was maintained at 20^{o} C and a pH of 6.0 ± 0.2 with automated monitoring and additions of KOH (0.01 M) and H_2SO_4 (0.01 M). Nutrient levels were monitored and adjusted every two days to maintain solution concentrations. Four hydroponic systems were available in the environmental chamber, and therefore four generations could be tested in one trial. For each trial, parental transgenic canola and *B. rapa* generations were grown as controls and compared to the growth response of two hybrid generations. The high N experiment was performed with all six-plant generations (canola, *B. rapa*, F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk), and the low experiment was performed with 4 generations (canola, *B. rapa*, F_1, and BC_1F_1).

The optimal and sub-optimal N experiments were conducted over 39-day periods. Five plants per generation (4 generations grown concurrently, 20 plants total) were randomly harvested at three-day intervals beginning on the 18th day post germination (8 harvests total). At each harvest, total leaf area for each plant was recorded with a Li-Cor 3100 Leaf Area Meter (Li-Cor Instruments, Lincoln, NE). Total leaf area was calculated as the sum of all

measurable leaves (those greater than 5.0 cm^2). Shoot and root tissues of each individual plant were separated into paper bags, and were dried in a convection oven at 60^o C for 72 h. Dry weights per tissue type were recorded, and shoot to root (S/R) ratio was calculated (shoot weight divided by root weight). The five plants in each harvest were bulked by shoot and root tissue. The bulked tissue was ground and homogenized in a grinding mill (Instrument), and analyzed for total N with an automated CHN analyzer (Perkin Elmer 2400; Perkin Elmer Corp., Norwalk, CT or FlashEA 1112 Elemental Analyzer, ThermoQuest Italia S.p.A., Rodano, Italy software: Eager 300 ver 1.01). Total N content per individual was determined by multiplying N concentration of the bulked tissue type by the dry weight of each individual (N concentration x tissue mass). Total N per individual was calculated by adding the N mass per tissue type (N mass shoot + N mass root). Nitrogen use efficiency (NUE) was calculated as follows (Total dry mass ÷ [N])(Siddiqi and Glass 1981). At each harvest date, ANOVA was utilized for evaluating generational differences for the growth indices, and Fisher's PLSD was used to determine where significant differences occurred between generations (StatView 5.0 for Windows, 1992-1998, SAS Institute Inc., Cary, NC).

Seed analysis

Average seed weights were determined by weighing 20 seeds per plant generation on an analytical balance. Seed volume was calculated by a displacement method. For each plant generation, 50 seeds were randomly selected from the initial seed bulk and divided into 5 groups. Each group of 10 seeds was dropped into a 1 ml graduated pipette filled with ethanol (EtOH), and the displacement of the group was recorded. The average seed displacement of each group was then used to determine the average seed volume for the plant

generation. Nitrogen concentration was determined by combusting 10 seeds per plant generation with an automated CHN analyzer, and the N concentration was used to determine average N content per seed ([N] x seed mass). Statistical analysis was performed as above.

Temperature growth experiment

The growth of canola and *Brassica rapa* was analyzed at three temperature levels; 14º/18º C, 18º/22º C, 22º/26º C. The temperature experiment was conducted in paired, 84-l hydroponic systems in three separate environmental chambers with an 8 hr day length. Each hydroponic system contained 20 plants (3 paired systems, 60 plants total per species). The nutrient solution was prepared to the same levels as the high N experiments. The pH of the nutrient solution was tested with a hand-held pH meter (Instrument) and reset daily to a pH of 5.8. The solution temperature matched that of the environmental chamber.

The temperature response experiment was conducted over a 24-day period. All plants were harvested, and leaf areas were recorded for 5 randomly selected plants per species per temperature level (30 total). Plants were dried as above, and dry weights were recorded. A calculated leaf area was determined allometrically for all plants by using the equation generated by regressing leaf area versus dry weight of the 5 plants collected per species at each temperature level.

Field experiment

Field level competition between transgenic hybrid generations and a model crop was analyzed by growing plots (1 m^2) of transgenic canola, *B. rapa*, BC_2F_2 transgenic and non-transgenic hybrids in a wheat field. The competition experiment was conducted at the

Central Crops Research Station, Clayton, NC, USA (35° 39'N 78° 27'W) from October 2001 through May 2002. Wheat (*Triticum aestivum* L. cv USG 3209) was planted in late October (Oct 19th) with a row spacing of ca. 15 cm. Wheat seeding rates were ca. 75 seeds per meter planted with a small row planter. A complete randomized block design was utilized, and four *Brassica* populations (canola GT1, *B. rapa* QC-2974, BC_2F_2 transgenic and non-transgenic hybrids) were transplanted in 1 m^2 plots three weeks after the wheat was sown. Eight replications of the *Brassica* plots were randomly placed within the field. In the spring of the following year, wheat dry weight and seed mass were recorded per block, and competition was quantified by comparing wheat yields.

Results

Plant breeding and description of plant genetics

In regards to genomic composition, *B. rapa* × canola hybrid populations progress through dynamic chromosomal arrangements that begin at an intermediate triploid (F_1) state between the two parental species, and result in a diploid composition similar to *B. rapa* by the reduction of canola chromosomes after multiple backcrosses (Figure 1). The ploidy level of the F_1 hybrids was shown to be triploid (AAC), with a 2n number of 29 chromosomes (Metz et al 1997, Halfhill et al 2002). After one backcross generation, the ploidy of the BC_1F_1 generation shifted toward the lower DNA content of *B. rapa*. However, the ploidy was not identical with *B. rapa*, and demonstrated that a small portion of the C genome (perhaps 1-2 chromosomes) was not lost in the first meiotic division. The BC_2F_1 generation continued the trend toward the loss of genetic material, and the ploidy was indistinguishable from the *B. rapa* parental species with a diploid number of 20 chromosomes. The diploid

composition was stable after the intermating of BC_2F_1 individuals (Halfhill et al 2003), and demonstrates that a diploid population can be reached after 3 generations of backcrossing.

Plant morphology in hydroponics

When grown in hydroponics, *Brassica rapa* and canola were morphologically distinct. The leaves of *B.* rapa were triangular with many tricomes, and the rosette was short with small internode distances between leaves. Canola leaves were round and smoother, and the stem was generally taller with greater internode distances compared to *B. rapa*. The number of leaves differed between the parental species, with *B. rapa* consistently producing more leaves than canola. The root morphology was similar between both species. The F_1 hybrid generation was morphologically intermediate between the two parental species, and backcrossed generations (BC_1F_1, BC_2F_1, BC_2F_2 Bulk) were morphologically similar to the *B. rapa* parent (data not shown).

Optimum growth response

The optimum growth experiment (high N) was conducted in two trials, with the parental species grown in both trials with two hybrid generations (trial 1, F_1 and BC_1F_1; and trial 2, BC_2F_1 and BC_2F_2 Bulk). All plant generations exhibited exponential growth. *Brassica rapa* accumulated greater total dry weight and leaf area compared to canola and the hybrid generations (Figure 2)(Fisher's PLSD, $P<0.05$). In trial 1, total dry weight initially deviated between the hybrid generations (F_1 and BC_1F_1) and B. rapa 30 days post germination (Figure 2, A), while leaf area diverged at day 24 (data not shown). At the end of the trial, F_1 and BC_1F_1 hybrids accumulated 73% and 66% of the *B. rapa* total dry weight,

respectively. Differences in leaf area were greater, with the F_1 generation accumulating 64% and the BC_1F_1 generation 57% of the *B. rapa* leaf area (data not shown). Canola and *B. rapa* growth indices diverged at similar dates as the hybrid generations. Canola and the hybrid generations exhibited similar growth rates in trial 1, with similar average leaf area and total dry weight at the end of the experiment.

The growth of all plants in trial 2 was delayed in comparison to trial 1 (Figure 2). For the first harvest date (day 18) of trial 2, the harvested plants were too small for accurate determination of leaf area, and were excluded from the study. *Brassica rapa* differed significantly from the BC_2F_1 and BC_2F_2 Bulk hybrid generations starting on day 27 for total dry weight (Figure 2, C) and day 24 for leaf area. The BC_2F_1 generation exhibited the lowest growth rates in comparison to the *B. rapa* parental line, with 54% dry weight and 59% leaf area of *B. rapa* at the end of the trial. The BC_2F_2 Bulk generation also differed from *B. rapa*, and accumulated 67% and 78% of the *B. rapa* total dry weight and leaf area. In trial 2, canola and *B. rapa* dry weights diverged later than trial 1 at day 33, and earlier for leaf area where the two species diverged at day 24. Canola generally exhibited similar growth characteristics to the hybrid generations, except total dry weight (Figure 2, C), in which canola consistently had greater dry weight than the hybrid generations.

N analysis and N use efficiency

Total N and N use efficiency were calculated for the optimal growth experiment. As was shown for the other growth indices, *B. rapa* exhibited the greatest accumulation of N when compared to the other generations (Figure 3, A and C). *B. rapa* diverged from the F_1 and BC_1 at day 30, and from the BC_2F_1 and BC_2F_2 Bulk generations at day 27. At the end of

the study, the hybrid generations consistently accumulated less N than *B. rapa*, and ranged from 75% (F_1 generation) to 50% (BC_2F_1 generation) compared to the *B. rapa* total N. In trial 1, canola and the F_1 and BC_1F_1 generations were generally similar with regard to total N. In trial 2, canola accumulated significantly more total N than hybrid generations BC_2F_1 and BC_2F_2 Bulk. Nitrogen use efficiency was calculated based on the N concentration in the shoot. The *B. rapa* parental species was found to have a higher N use efficiency than any of the four hybrid generations (Figure 3, B and D). In both trials, *B. rapa* and canola demonstrated similar values until day 30, and then diverged at day 33 with *B. rapa* presenting higher N use efficiencies. The hybrid generations consistently had lower N use efficiencies than the parental generations in both trials.

Sub-optimal growth response

The sub-optimal growth experiment was conducted with *B. rapa*, F_1 hybrid, BC_1F_1 hybrid, and canola. Differences in total dry weight and leaf area among the plant generations were not found to be significant (Fig. 4). At the first collection date, the leaves were too small for accurate determination of leaf area, but dry weights were recorded and included in the analysis. *Brassica rapa* and canola grew at similar rates throughout the experiment, but differences were detected between the parental and hybrid generations during the 24-30 day interval (Fig 4, A). During this period, the parental lines accumulated significantly more dry weight than the hybrid generations (Fisher's PLSD, $P<0.05$). These differences were overcome by day 33, and dry weight and leaf area remained similar between all four-plant generations until the end of the experiment.

Shoot to root ratio

Shoot to root (S/R) ratio was determined for all plants under optimal and sub-optimal conditions (Figure 5). Under optimal conditions, the S/R ratio of all lines increased during the 39-day experimental period. At each harvest, variation was high for this index, but all generations exhibited an increasing trend (Figure 5, A). Canola initially exhibited a higher S/R ratio than *B. rapa*, but this difference was reduced by day 30 and the ratios were consistent for the rest of the experiment. The hybrid generations were intermediate between the parental species, but varied greatly with no significant differences detected. The S/R ratio for the sub-optimal growth treatment differed from the optimal treatment, with a general trend for a reduced S/R ratio (Figure 5, B).

Seed analysis

Four different seed characteristics were quantified for the six plant generations, and significant differences were detected among plant generations (Table 1). Canola exhibited greater seed volume and seed weight than the other five lines in the study, which were not significantly different from each other. All seeds harvested from *B. rapa* parents were 57.5% of the canola seed volume and 45.5% of the canola seed weight. For N concentration, a range of concentrations was determined between the plant lines. The N concentration was not consistent amongst the hybrids, and significantly varied from one another ranging from 2.87% of the F_1 generation to 4.99% for the BC_2F_2 Bulk. The parental lines were intermediate between the hybrid generations, with canola having an N concentration of 3.49% and *B. rapa* containing 3.14% N. The N concentration was then used to calculate seed N content, and canola contained significantly more N per seed than the other generations. *B.*

rapa and the hybrid generations once again varied. *B. rapa* and the earlier hybrid generations (F_1 and BC_1F_1) had significantly less N per seed than the later generations (BC_2F_1 and BC_2F_2 Bulk).

Temperature growth response

The parental species were tested under a range of temperatures in order to determine if species-specific temperature optima was a confounding factor in the optimal and sub-optimal growth experiments. After 21 days of growth, both species showed a growth response directly proportional to increasing temperature (Figure 6). In both cases, as temperature increased, the rate of growth increased. Over the temperature range, canola exhibited higher total dry weight and calculated leaf area compared to *B. rapa*. In this experiment, both species exhibited a similar growth response, and therefore temperature did not seem to be a confounding factor in the differences detected in growth potential.

Field experiment

Under field conditions, the *Brassica* plants established successfully and grew within the wheat field. The *Brassica* plants behaved as summer annuals, and began flowering in early March (ca. 140 d post germination). The *Brassica* plants reached a height ca. 0.5 m taller than the stand of wheat (data not shown). *Brassica* flowering was completed by April 11 (ca. 175 d post germination). The wheat field was allowed to mature, and the $1m^2$ plots were harvested on May 9th (ca. 205 d post germination).

In all experimental plots, *Brassica* competition caused a reduction of wheat seed mass and dry weight (ANOVA, $P < 0.05$)(Figure 7). When wheat growth and yield were analyzed

among the *Brassica* generations, transgenic hybrids were the least competitive. Canola, *B. rapa*, and non-transgenic BC_2F_2 Bulk hybrid populations reduced wheat seed mass by 46.0%, 48.2%, and 44.5%, respectively. The transgenic BC_2F_2 Bulk generation had significantly less effect on wheat seed mass with an average reduction of 25.5% (Fishers PLSD, $P < 0.05$). Statistically similar results were found for wheat dry weight.

Discussion

The results of these experiments demonstrate that transgenic hybrid generations had low potentials for growth and competitiveness with a crop under field conditions. *B. rapa* demonstrated the highest potential vegetative growth rates when compared to transgenic hybrid generations. Hybrid generations did not show increased growth rates during the early vegetative stage when canopy development occurs. The physiology of the hybrid generations was altered in comparison to the wild species, and growth rates were consistently reduced from the triploid F_1 generation to the *B. rapa*-like diploid BC_2F_2 Bulk generation. Growth potential was not restored to the *B. rapa* level with a return to the normal, diploid chromosome number (2n=20) of the wild species. Throughout the experiments, the hybrid generations demonstrated similar growth potentials as canola. Therefore, the backcrossed hybrid generations had the morphology of the weedy *B. rapa* parent, but the physiological characteristics of the crop *B. napus* parent. This may represent a diminished capacity for transgenic hybrids to compete with the same efficiency as the weed-like parent, and therefore may represent a reduced risk in the production of a more competitive, transgenic weed.

The experiments reported here analyzed vegetative growth potential and competitiveness, and represent a departure from other risk assessment studies that typically

focused on the measurement of reproductive ability. In a previous report, Hauser et al. (1998, a) found that F_1 hybrid individuals had intermediate fitness between canola and *B. rapa* based on several combined characteristics, and they concluded that F_1 hybrids would generally be more fit than *B. rapa* based on mostly reproductive indices. In a subsequent study, it was found that a fitness depression occurred in F_2 and backcrossed individuals, but they found a small percentage hybrids were as fit as the parents (Hauser et al 1998, b). In contrast, our work focused on hybrid vegetative growth potential and the ability to negatively impact crop yield by direct competition. From our work, we found that the weedy *B. rapa* parent had the highest growth parameters, and therefore, the greatest risk to crop production would be the selection for hybrid generations that had the had similar growth characteristics as the *B. rapa* parent. Weedy species generally respond in high nutrient environments with increased growth rates compared to competing crop species (Appleby et al. 1976, Liebl and Worsham 1987), and the hybrids generations tested in these studies did not show a weed-like growth under optimal conditions. The physiological characteristics of hybrid populations will cause negative impacts crop production, and therefore, we believe that growth and competitiveness should be an equal or larger focus than reproductive factors in risk assessment studies

The selection for transgenic individuals, as was done in this report, did not generate populations with high vegetative potential. This could by influenced by several factors, including the degree of genetic co-introgression of crop-adapted genes in hybrid generations and the presence of transgenes. The degree of *B. napus* genetic introgression into multiple backcross hybrid generations with *B. rapa* has been quantified using AFLP analysis. AFLP analysis generates dominant markers based on the presence or absence of specific DNA

sequences, and the F_1 hybrid generations contained 95-97% of the *B. napus*-specific AFLP markers. Subsequently, each successive backcross generation demonstrated a reduction of markers resulting in a 15-29% presence in the BC_2F_2 Bulk population (Halfhill et al. 2003). As the genetic composition of the populations returned to the diploid state, a substantial amount of crop genetic material was introgressed into the hybrid genetic background. The physiological changes characterized for the hybrid generations may be caused by the introgression of crop adapted (*Brassica napus*) genetic material, and may illustrate that hybrids may not pose the same risk in regards to competitiveness as the wild parental species.

The presence and expression of transgenes could also play a role in the reduced fitness of these transgenic hybrid populations. The GFP and Bt transgenes may affect physiological processes by interrupting endogenous genes and by producing proteins that interfere with the normal biochemical pathways. Anecdotal evidence has been reported that GFP may have some cytotoxic effects in some transgenic events (Hasseloff et al 1997), although conflicting evidence to this conclusion have been reported. When GFP tobacco was grown under field conditions, Harper et al. (1999) found that GFP production did not correlate to a reduction in plant biomass and seed production. The Bt protein produced by these transgenic hybrid generations has been produced in several transgenic varieties of field crops, such as cotton and maize, and specific reductions in yields have not been demonstrated by the production of this protein, and to the contrary, yield increases have been detected under agronomic conditions (Graeber et al. 1999, Magg et al. 2001, Pray et al. 2002, Qaim and Zilberman 2003). Previous studies have analyzed multiple fitness components of transgenic hybrid generations from the crossing of herbicide tolerant canola and *B. rapa*. Snow et al. (1999) analyzed reproductive indices and found that there were no significant

differences between transgenic and non-transgenic BC_3 plants in seed production. They concluded that the cost associated with the herbicide tolerance transgene is negligible to hybrid populations based on reproductive indices. Although specific physiological costs of the GFP and Bt transgenes were not tested in the current report, it is unlikely that the reduction of growth potential and competitiveness in hybrid generations was caused by the production of the recombinant proteins. The effect of the insertion locus of the transgene cassette in the original transgenic canola event will be evaluated in future field experiments.

The questions of increased fitness and ecological impacts of transgenic hybrid populations under field conditions are the logical next step in risk assessment, and current research provides important baselines to focus the questions of future research. The findings of this report may underestimate the risks posed by transgenic hybrid populations under field conditions. The hybrid populations produced in this study were selected solely based on the presence or absence of the GFP phenotype. Under field conditions, natural selection should be expected to exert selective pressure on these populations and shift the genetic complements toward adapted genotypes as a hybrid population progresses through generations. The resultant introgressed population may utilize the most adapted genes from either parent, and may result in a population that has been selected for similar competitiveness as the wild parent. Also, the experiments presented here did not add any selective advantage for the transgenic phenotype, and therefore may underestimate the competitiveness of transgenic hybrids when selection pressure is present. Future experiments must be performed under field conditions that incorporate selection pressures for added transgenic phenotypes in order to assess the specific risks of transgenic hybrids.

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Table 1. Seed analysis (means ± SE) of parental and transgenic hybrid generations. Different superscripts represent significant differences between means (Fisher's PLSD, $P < 0.05$).

Generation	Seed Weight (mg)	Seed Volume (μl)	N Concentration (%)	Seed N Content (mg)
Canola	4.94 ± 0.09^{a}	4.02 ± 0.21^{a}	3.49 ± 0.08^{a}	0.17 ± 0.010^{a}
Brassica rapa	2.25 ± 0.08^{b}	2.53 ± 0.12^{b}	3.14 ± 0.21^{ab}	0.07 ± 0.009^{b}
F_1	2.27 ± 0.11^{b}	2.46 ± 0.12^{b}	2.87 ± 0.08^{b}	0.07 ± 0.006^{b}
BC_1F_1	2.26 ± 0.09^{b}	2.25 ± 0.09^{b}	3.34 ± 0.13^{a}	0.08 ± 0.007^{b}
BC_2F_1	2.31 ± 0.08^{b}	2.22 ± 0.16^{b}	4.54 ± 0.12^{c}	0.11 ± 0.008^{c}
BC_2F_2 Bulk	2.16 ± 0.07^{b}	2.10 ± 0.19^{b}	4.99 ± 0.11^{d}	0.11 ± 0.007^{c}

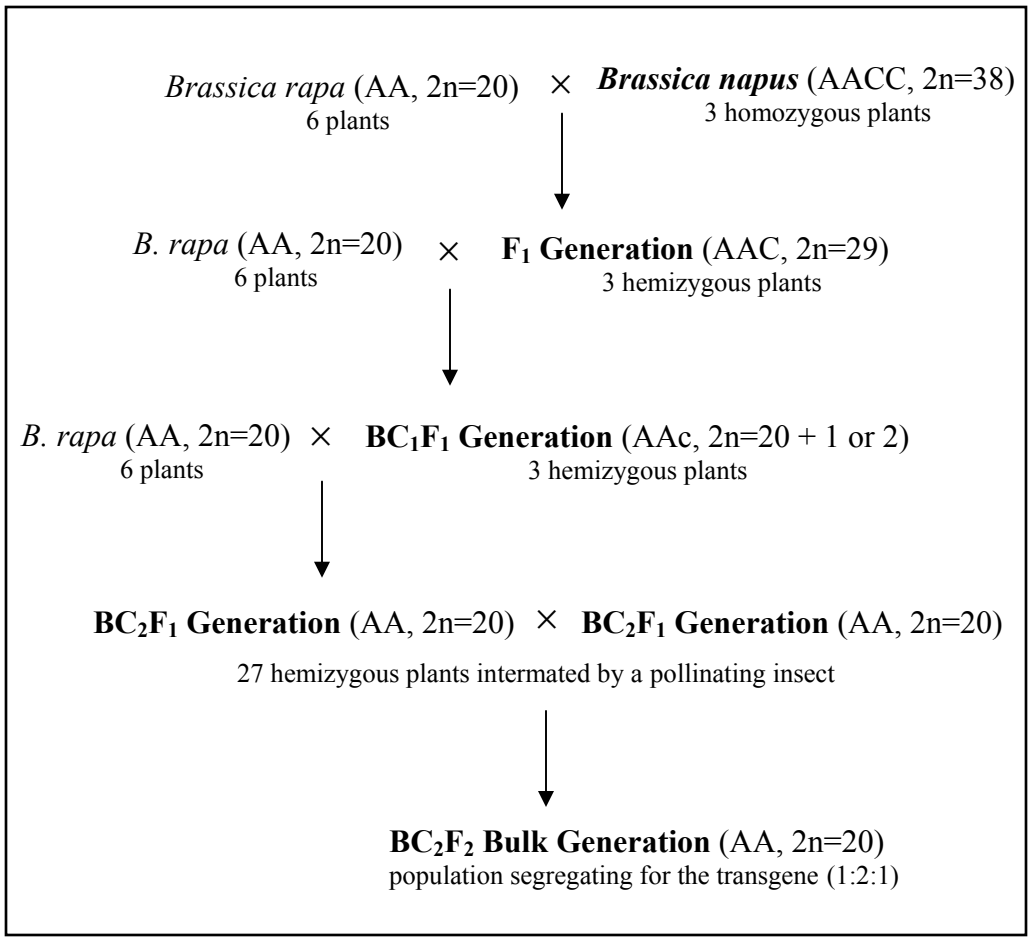

Figure 1. The plant breeding pattern used to produce the hybrid generations utilized in the growth analysis experiments. The maternal parent was represented in the first position in each cross, and generations in bold were transgenic. In the parenthesis, the letters represent genomic composition of the generation, and the 2n number represents the estimated chromosome number (Halfhill et al. 2002 and Halfhill et al. 2003). The number under the generation represents the number of plants crossed to produce the subsequent generation. The BC_2F_2 Bulk generation was a segregating population consisting of 25% homozygous, 50% hemizygous, and 25% isogenic for the transgene.

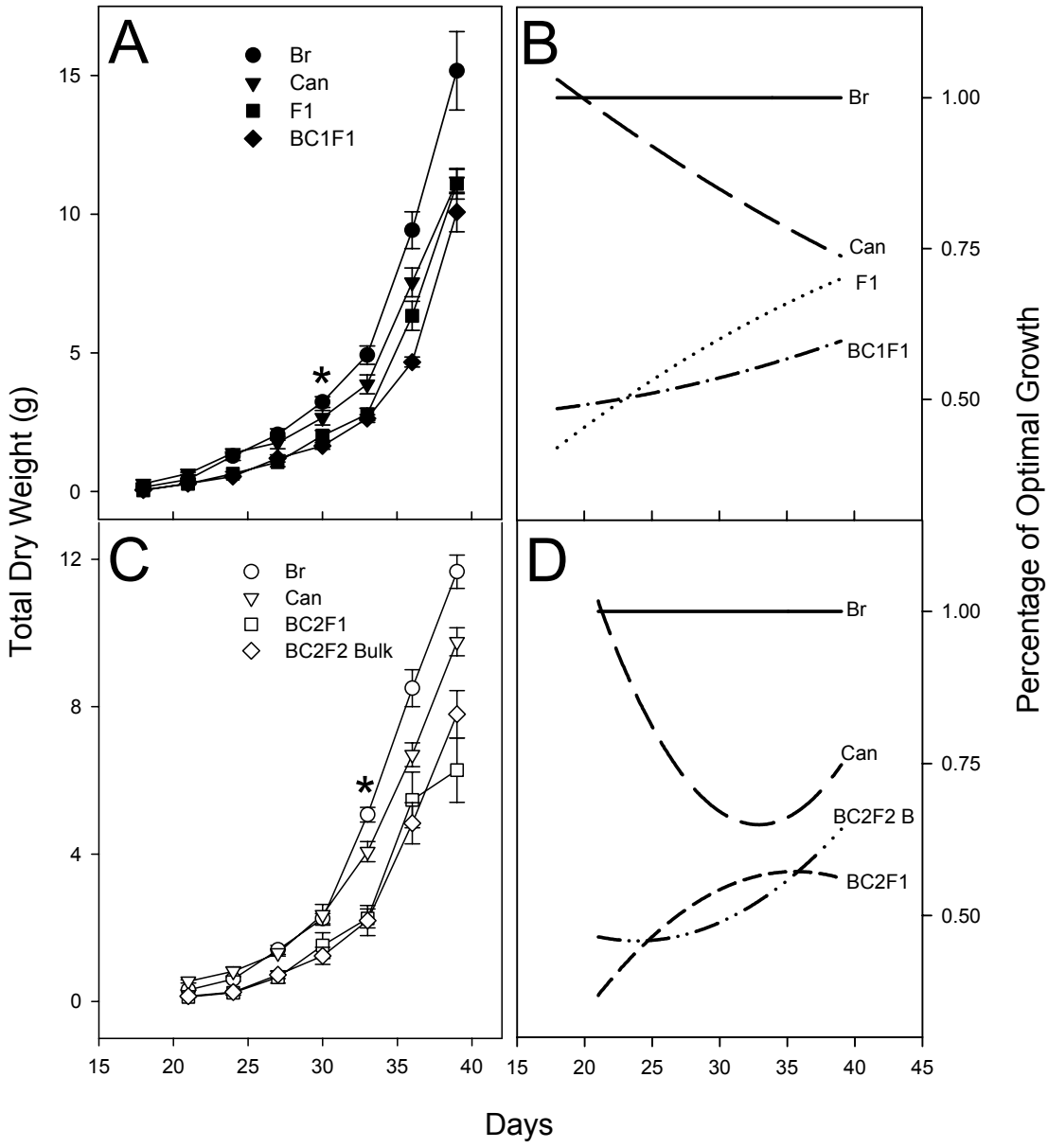

Figure 2. Total dry weight and percentage of optimal growth of parental and hybrid generations under optimal conditions (high N). Panels A and B represent trial 1, and Panels C and D represent trial 2. The asterisk (*) represents the initial date when *Brassica rapa* (Br) significantly differed from canola (Can) and the hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk)(Fisher's PLSD, $P<0.05$). Canola generally exhibited similar growth characteristics at each harvest date as the hybrid generations, except total dry weight in panel C, where canola consistently differed from the hybrid generations.

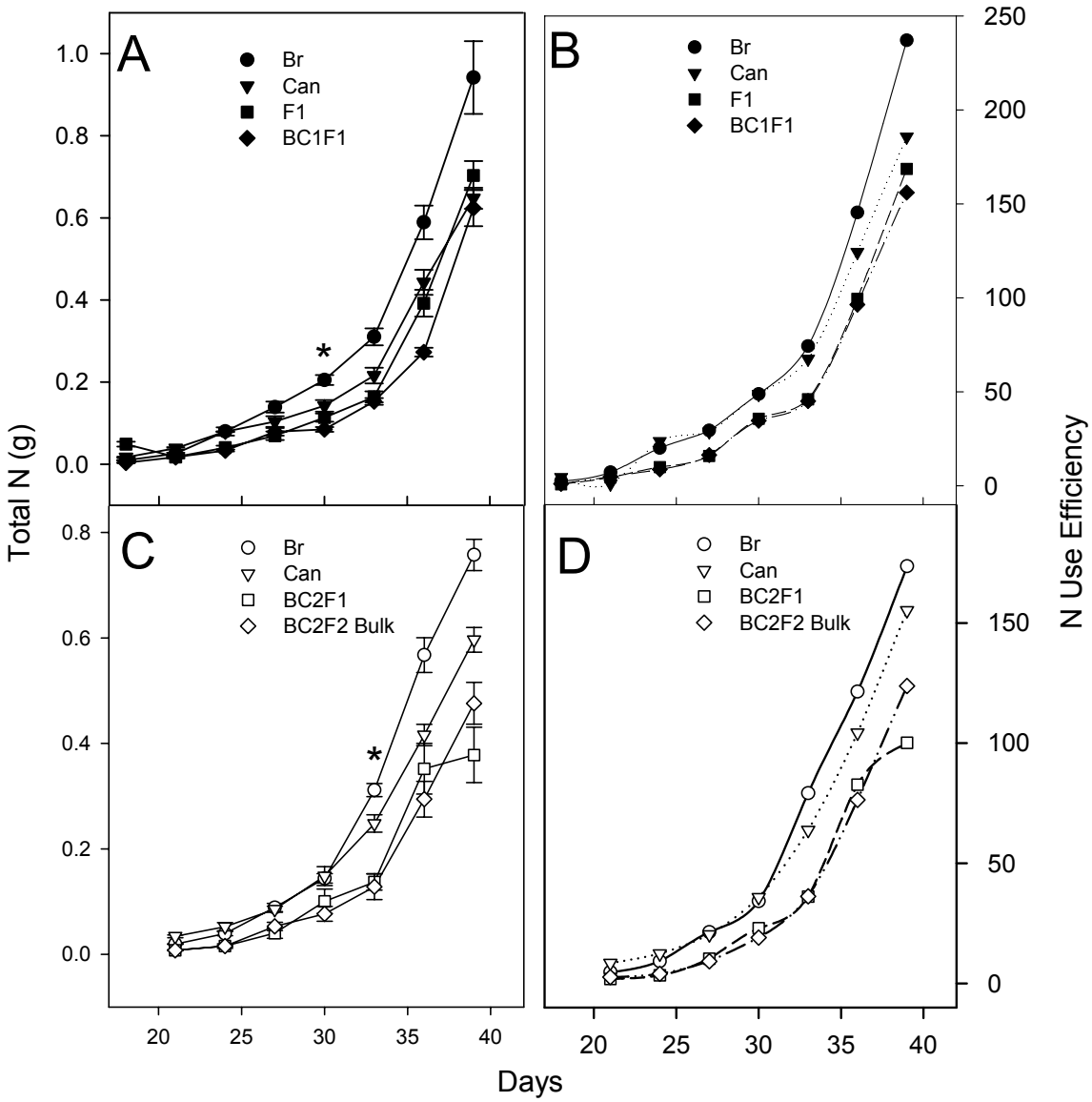

Figure 3. Total N and N use efficiency of parental and hybrid generations under optimal conditions. Panels A and B represent trial 1, and Panels C and D represent trial 2. The asterisk (*) represents the initial date when *Brassica rapa* (Br) significantly differed from canola (Can) and the hybrid generations (F_1, BC_1F_1, BC_2F_1, and BC_2F_2 Bulk)(Fisher's PLSD, $P<0.05$). The parental species (Br and Can) consistently demonstrated higher N use efficiency when compared to hybrid generations.

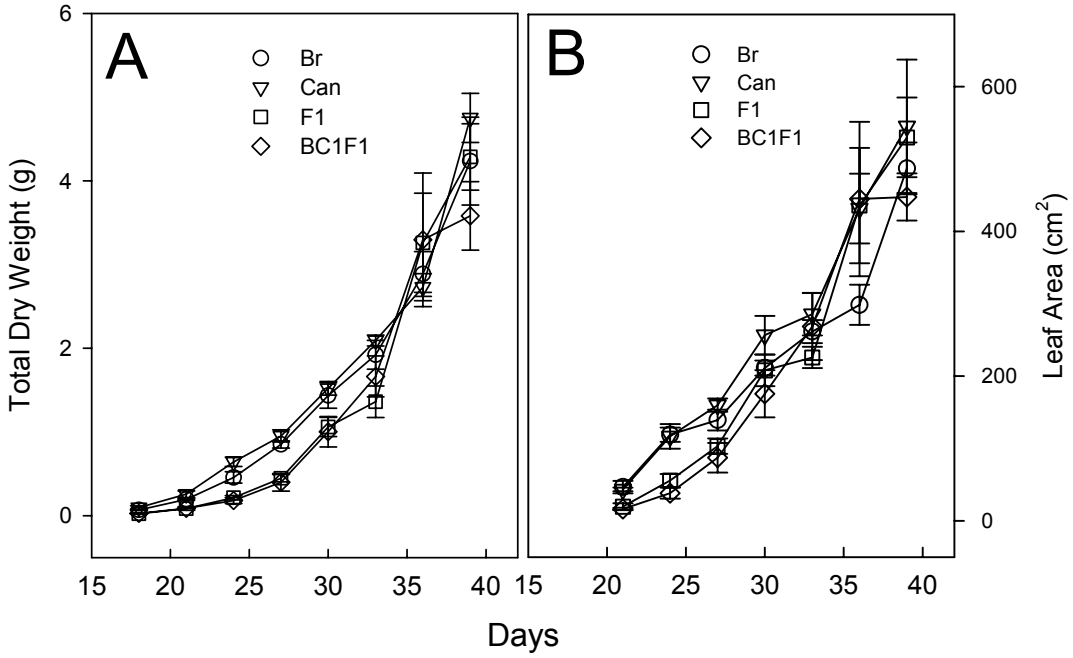

Figure 4. Total dry weight and leaf area of parental (*Brassica rapa,* Br; and canola, Can) and hybrid generations (F_1 and BC_1F_1) under sub-optimal conditions (low N). Growth characteristics were generally similar between all generations.

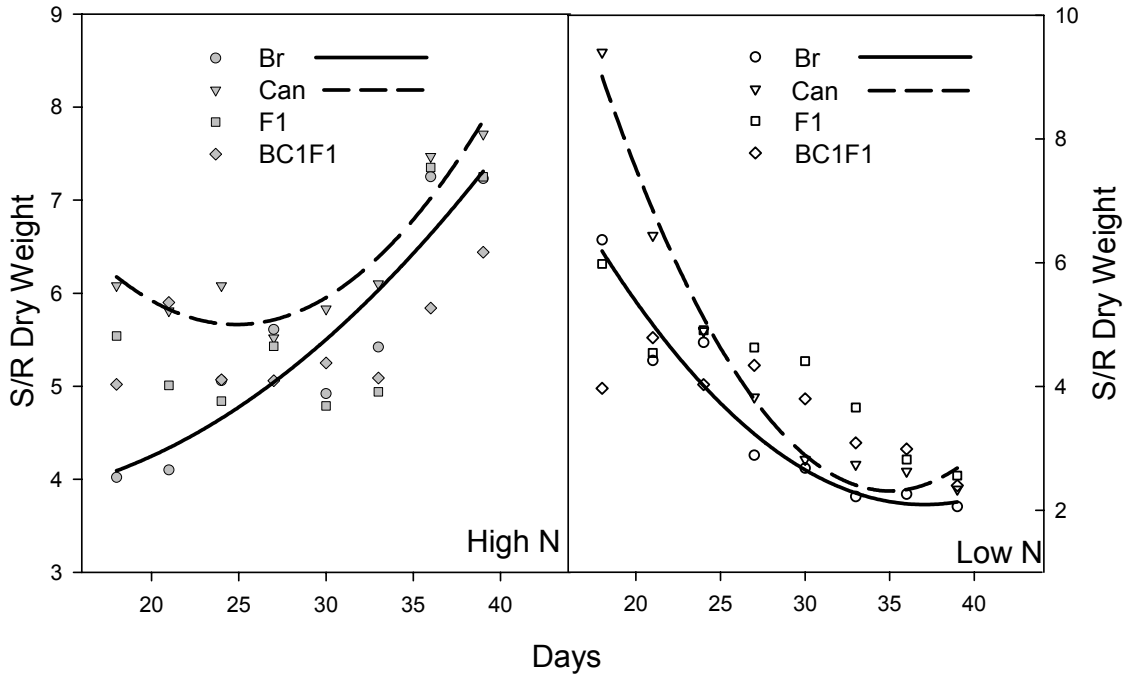

Figure 5. Shoot to root ratio of parental and hybrid generations under optimal and sub-optimal conditions. All plant generations, shoot to root ratio increased under high N, and decreased under low N.

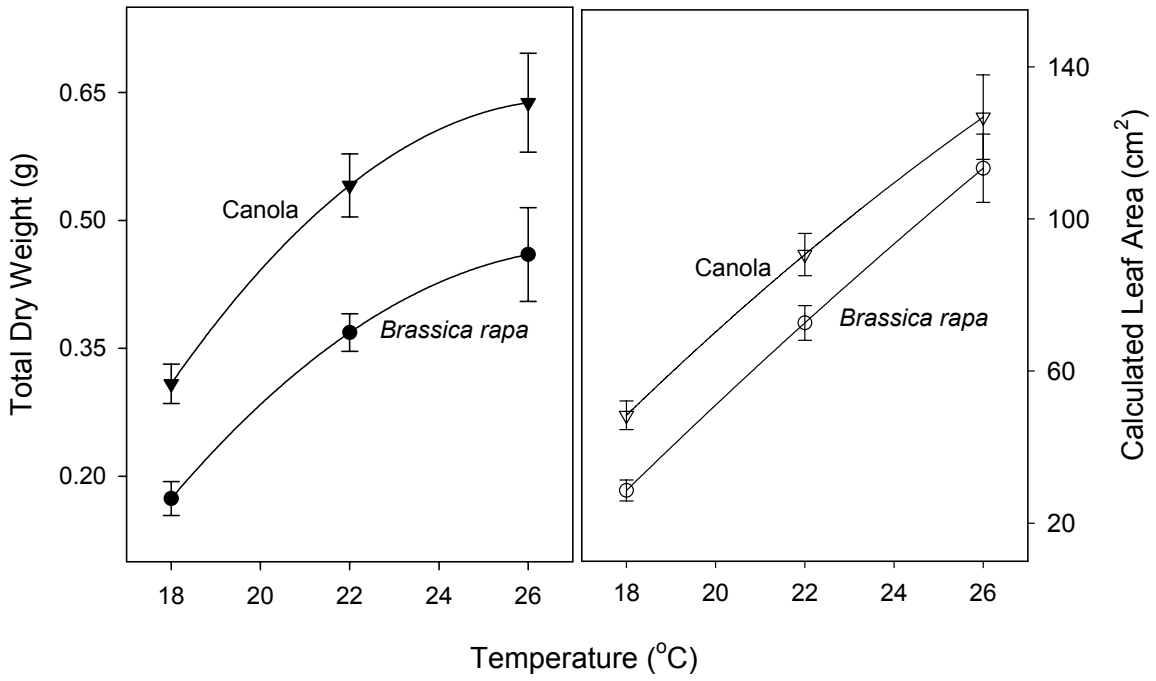

Figure 6. Temperature response of canola and *Brassica rapa* when grown for 24 days under optimal conditions (high N). Growth response was directly proportional to increasing temperature.

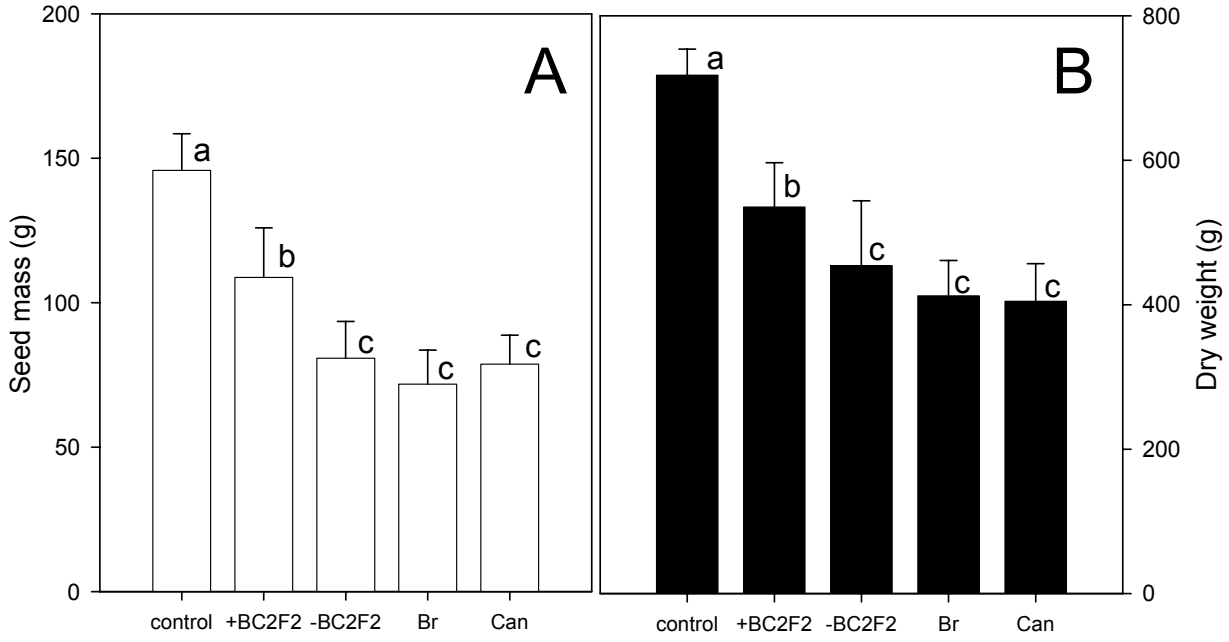

Figure 7. Wheat seed mass (A) and total dry weight (B) when grown in competition with several *Brassica* lines. Five treatment types are presented: control, wheat only; +BC2F2, transgenic BC_2F_2 hybrids; -BC2F2, non-transgenic BC_2F_2 hybrids; Br, *Brassica rapa* 2974; and Can, transgenic canola line GT 1. Different letters represent significant differences between treatments (Fisher's PLSD, $P<0.05$).

Chapter 7: Gene flow from transgenic *Brassica napus* L. and crop/weed hybrids with relative weeds under field conditions

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Abstract

Hybridization between genetically modified crop varieties and wild relatives is a biological possibility. Determining the frequency of transgene flow under field conditions is a fundamental necessity for the development of regulatory strategies that manage transgenic hybrids. Gene flow of green fluorescent protein (GFP) and *Bacillus thuringiensis* (Bt)

transgenes was quantified in three field experiments using eleven genetically modified *Brassica napus* L. (canola) events and the wild relatives, *Brassica rapa* L. and *Raphanus raphanistrum* L. Under a high crop to wild relative ratio (600:1), hybridization frequency with *B. rapa* differed among transgenic *B. napus* events (ranging from ca. 4% to 22%), and demonstrated an average frequency over all events of ca. 10%. No hybridization with *R. raphanistrum* was detected. Under a lower crop to wild relative ratio (180:1), hybridization frequency with *B. rapa* was consistent among the *B. napus* events at ca. 2%. Differences of hybridization frequency among wild relatives within and at the periphery of a genetically modified crop field were quantified, and hybridization frequency was higher when *B. rapa* occurred within the *B. napus* field (ca. 37.2%) compared to the periphery of the field (ca. 5.2%). No significant differences were detected among peripheral plants grown at 1, 2, and 3 m distances from the field. Backcrossing frequency between transgenic hybrids and *B. rapa* was determined in two field experiments in which the wild relative to transgenic hybrid ratio was between 5:1 and 15:1. As expected, ca. 45% and 50% of the seeds produced from the hemizygous hybrid plants were transgenic backcrosses in the two experiments, respectively. When *B. rapa* plants served as the maternal parent, backcrossing frequencies were much lower at 0.088% and 0.060% in experiments 1 and 2, respectively. From these field experiments, it is clear that transgene flow from many independent transgenic events of *B. napus* to *B. rapa* can occur under a range of field conditions, and maternal transgenic hybrids will produce higher numbers of transgenic backcrosses compared to the seeds produced from wild relatives. This suggests that regulatory efforts should focus on maternal F_1 transgenic hybrids as the source with the greatest potential to generate transgenic wild-relative populations.

Keywords: transgenic canola, GFP, interspecific hybridization, *Brassica rapa, Bacillus thuringiensis*, introgression

Introduction

Outcrossing of genetically modified crop varieties to wild relatives and the transfer of novel phenotypes to wild populations is a concern that must be addressed by regulatory management to mediate potential risks posed by unwanted hybrid populations. The biological possibility of hybridization has been confirmed in many laboratory and field based experiments, and future research should determine the frequency of hybridization under a wide range field conditions and transgenic varieties. The potential for ecological and agricultural risks are based on the frequency of the occurrence of transgenic hybrid populations, and therefore, the next step in risk assessment studies must quantify hybridization frequency in order to develop regulatory strategies that manage transgenic hybrids.

Canola (*Brassica napus* L.) is of particular interest as a potential source for transgene escape due to several factors. Large percentages of genetically modified (GM) varieties are grown in geographic regions with commercial canola production (60% of Canadian *B. napus* production is GM, Warwick et al. 2003). Canola production leaves significant volumes of seed in the field due to shattering and harvesting procedures. Lost seeds result in large volunteer populations that may remain for several years (Légère et al. 2001, Simard et al. 2002). Canola pollination occurs by both selfing and outcrossing (average outcrossing frequency 30%, reviewed in Beckie et al. 2003). Canola volunteer populations have been shown to be resistant to multiple herbicides as the result of intraspecific hybridization (Hall

et al. 2000, Beckie et al. 2003). In many areas of canola production, wild relatives such as *Brassica rapa* (birdseed rape, field mustard), *Raphanus raphanistrum* (wild radish), and *Sinapsis arvensis* (wild mustard) occur near areas of cultivation. In a current report, Beckie et al. (2003) have demonstrated the transfer of an herbicide (glyphosate) resistance gene from commercial canola production to a naturally occurring population of *B. rapa* in Quebec, Canada. The agronomic characteristics of canola production combined with the occurrence of sexually compatible wild relative species near areas of canola production demonstrate the necessity to monitor canola as a potential source of transgenes to wild populations, at least until the consequences can be determined.

The introgression of transgenes into transgenic hybrid populations occurs in a step-wise fashion starting with the initial hybridization between the transgenic crop and the wild relative. The frequency of hybridization may potentially be affected by many factors, including the transgene insertion locus within the transgenic event (Halfhill et al. 2002), the agronomic properties of transgenic variety, the crop to wild relative ratio, the distance between the transgenic crop and wild relative, and the environmental conditions under field conditions. In this study, three field experiments were performed to quantify hybridization between multiple transgenic canola events and two wild relatives (*Brassica rapa* and *Raphanus raphanistrum*) under a range of field conditions. The effect of crop density was determined by performing experiments with high and low crop to wild relative ratios. Growing wild relatives at three adjacent distances to a transgenic field compared hybridization rates between wild relatives within and at the periphery of the field. Backcrossing rates of transgenic hybrids was quantified to determine the incidence of introgression during subsequent generations. Two backcrossing experiments were performed

to determine the frequency of backcrossing between a transgenic hybrid and a population of wild relatives. Together, these experiments are among the first to comprehensively characterize hybridization and backcrossing frequencies among different transgenic events, sites, and years.

Materials and methods

Plant material

Primary transgenic events (T_0) of canola (*Brassica napus* cv. Westar) transformed with a plasmid containing GFP (*Aequorea victoria, mGFP5er* variant, Haseloff et al. 1997) and Bt (*Bacillus thuringiensis,* synthetic Bt *crylAc*) genes received the label "GT", and plants transformed with a plasmid containing only GFP were labelled "GFP" (Halfhill et al. 2001). A transgenic event was defined as the progeny of a single, independently transformed plant recovered from tissue culture. Nine homozygous T_3 GT (GT 1-9) events and three homozygous T_3 GFP (GFP 1-3) events were used as model transgenic crop varieties. Two weed species were selected as model wild relatives: *B. rapa* and *Raphanus raphanistrum.* Three accessions of *B. rapa* (*Br* CA from Irvine, California, USA (33° 40'N 117° 49'W), courtesy of Art Weis; *Br* QC-2974 from Milby, QC, Canada (45° 19'N 71° 49'W) and *Br* QC-2975 from Waterville, QC, Canada (45° 16'N 71° 54'W) germplasm collection AAFC-ECORC, Ottawa) and one accession of *R. raphanistrum* (*Rr* GA from Leesburg, Georgia, USA (31° 44'N 84° 10'W), collected as separated seed from a rye seed cleaning facility), were used in these field experiments.

Hybridization field experiments

Three field level F_1 hybridization experiments were conducted, one with a high crop to wild relative ratio (600:1), the second with a low crop to wild relative ratio (180:1), and a third that compared hybridization within and on the periphery of transgenic crop field. Two backcrossing experiments were performed with a wild relative to transgenic hybrid ratio between 5:1 and 15:1.

High *Brassica napus* to wild relative ratio (600:1)

A GFP *Brassica napus* field experiment was conducted at Central Crops Research Station, Clayton, NC, USA (35° 39'N 78° 27'W) in the spring of 2001 with a *B. napus* to wild relative ratio of 600 to 1. Ten events of transgenic GFP *B. napus* (GT 1-5, GT 8-9, and GFP 1-3) were hand planted in ten 2 m × 48 m plots, one event per plot. The seeds were randomly scattered with a planting rate of 100 seeds per m^2 for a final stand density of 50 plants per m^2 (50% expected germination rate). At the time of canola planting (March 27, 2001), 16 *B. rapa* CA and *R. raphanistrum* GA seeds were sown at 6-meter intervals in the center of each plot. After germination, the *B. rapa* and *R. raphanistrum* plants were culled to 1 plant per 6-meter interval, resulting in 8 wild relatives per plot. Time of flowering was recorded for *B. napus*, *B. rapa*, and *R. raphanistrum.* At maturity, seeds from each wild relative were collected individually in paper bags, cleaned, and placed in a 4° C refrigerator to break dormancy. *Brassica rapa* seeds and *R. raphanistrum* pods were germinated in trays under greenhouse conditions, and all seedlings were screened at the cotyledon stage 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). Hybridization

rates were calculated by observing the number of plants that were green fluorescent (transgenic) compared to the total number of germinated seedlings as described by Halfhill et al. (2001).

Low *B. napus* to wild relative ratio (180:1)

A GFP *B. napus* field experiment was conducted at the Upper Piedmont Research Station in Reidsville, NC, USA (36° 20'N 79° 39'W) in the spring of 2000 with a crop to wild relative ratio of 250:1. Five homozygous events of transgenic *B. napus* (GT 1, 5, 8 and GFP 1-2) were planted in 5 m × 5 m blocks (1 event per block, 38 blocks total). Each 25 m^2 block was randomly planted within the field, and the blocks were separated by a 5 m boundary on all sides. Canola seeds were hand-planted in early March 2000 at a rate of 20 seeds per m^2 for a final stand density of 10 plants per m^2 (50% expected germination rate). Sixteen *B. rapa* CA seeds were planted at the center of each block at the same time as the *B. napus* events, and were later culled to 1 wild relative plant per block. Seeds were harvested in paper bags in early June from the *B. rapa* CA plants, cleaned, and placed in a 4° C refrigerator. Seeds were germinated in trays in controlled environmental chambers. Seedlings were screened at the cotyledon stage by visual, qualitative assay for GFP fluorescence. Hybridization rates were calculated in the same fashion as above.

Periphery experiment

A GFP *B. napus* field experiment was performed at the Central Experimental Farm, AAFC-ECORC, Ottawa, Canada (45° 23'N 75° 43'W) in 2001. In a 10 m × 10 m block (100 m^2), seeds of GT 9 *B. napus* were sown in 31 rows with the distance of 0.33 m between rows

with a planting rate of 160 seeds per m^2. Within the field, one seed of *B. rapa* QC-2975 was sown or one plant transplanted starting at the 0 m or block edge, with the distance of 1m between rows (11 rows total) and 1m apart between plants in each row (11 plants total per row). Thus, a total of 121 *B. rapa* plants were planted within the block. To test the impact of distance of pollen flow on the hybrid rate at the periphery of the field block, on the east side of the field block, *B. rapa* QC-2975 plants were planted or transplanted in rows at distances of 1m, 2m, and 3m respectively at the periphery of the field block (33 *B. rapa* plants total). *Brassica rapa* QC-2975 plants were allowed to mature in the field, and seeds of each *B. rapa* plant were harvested individually. Seeds were then cleaned, germinated under greenhouse conditions, and screened for GFP expression at 2-3 week old stage as above.

Backcrossing experiments

Backcrossing frequency was quantified in two field experiments at the Central Crops Research Station, Clayton, NC, USA (35^o 39'N 78^o 27'W) in 2000-2001, and at the Lang Research Farm, Tifton, GA, USA (31^o 27'N 83^o 30'W) in 2001-2002, respectively. The fields were planted in November in each location, and were completed in May of the following year. In NC, a total of 15 different transgenic F_1 hybrids from the hybridization of *B. rapa* accessions CA, QC-2974, and QC-2975 and *B. napus* events GFP1, GT1, GT4, GT5, and GT7, respectively and two BC_1 hybrids from *Br* CA x GT1 and *Br* QC-2974 x GT4 were utilized. In each 1 m × 1 m plot, one transgenic F_1 or BC_1 hybrid was surrounded by the respective *B. rapa* parental accession. Three replicate plots from each canola event by *B. rapa* accession were planted (example: 3 CA × GT1 plots, 3 QC-2974 × GT1 plots, and 3 QC-2975 × GT1 plots) for a total of 45 F_1 plots and 6 BC_1 plots. In GA, F_1 and BC_1 hybrids

from the cross of *B. rapa* CA and *B. napus* events GFP2, GT3, and GT8 were planted. Five replicate plots of each F_1 and BC_1 hybrid type were planted (30 plots in total). Within each 1 m × 1 m plot, 50 *B. rapa* seeds of the parental accession were hand-planted randomly over the plot, and 16 putative transgenic hybrid seeds were hand-planted at the center of each plot in early November. After germination, transgenic hybrids were screened at night for GFP fluorescence with a hand-held UV light and culled to 1 transgenic hybrid per plot. The *B. rapa* plants were culled to 15 plants per plot, leading to a wild relative to transgenic hybrid ratio of 15 to 1. At maturity in the following spring, the seeds of each plant (both the transgenic hybrid and *B. rapa* plants) were collected individually in paper bags from each plot and cleaned. Seeds were germinated on moist filter paper and screened for GFP fluorescence, and hybridization frequency was calculated as above.

Results

High *B. napus* to wild relative ratio (600:1)

In the high *B. napus* to wild relative ratio, *B. napus* and *B. rapa* CA (*Br* CA) germinated at expected rates, resulting in 50 canola per m^2 and 1 *Br* CA per 6 m interval. *R. raphanistrum* GA (*Rr* GA) demonstrated variable germination rates, and resulted in fewer than 8 *Rr* GA plants per plot with some 6m-intervals lacking a *Rr* GA plant. The range of *Rr* GA plants per plot were between 2 and 5 plants (33 GA plants total). Canola, *Br* CA, and *Rr* GA behaved as summer annuals, with *Br* CA flowering first on May 10. The canola events flowered a week earlier (May 17) than *Rr* GA (May 24). At the time of flowering, *Br* CA plants were similar in size to the canola. *Raphanus raphanistrum* GA plants were slightly shorter than the canola canopy, but *Rr* GA plants had more branches resulting in a larger

aerial circumference than an average canola plant. Flowering between the three species overlapped for 4 weeks. Canola and *Br* CA finished flowering 5 weeks after the initial flower was recorded, while *Rr* GA continued to flower to the date of collection (June 25). The *Rr* GA plants matured in stages, with mature seedpods present on flowering plants. This was in contrast to *Br* CA plants that had a well-defined period of flowering then finished prior to canola.

The seeds produced from the *B. rapa* CA maternal parents were germinated, and high germination rates were observed (data not shown). A total of 12,388 seedlings were screened for GFP fluorescence, and the average hybridization frequency over all *Br* CA seedlings analyzed was 9.66% (Table 1). Hybridization rates varied with transgenic *B. napus* event (Table 1)(ANOVA, $P < 0.01$). The highest hybridization frequency was found with *B. napus* event GT 5, with an average hybridization frequency of 22.0 ± 17.2 % (± standard deviation), and the lowest was found with *B. napus* event GT 8, with an average of 4.0 ± 3.3%. Hybridization frequencies from *Br* CA grown within canola events GFP 2 and GT 5 differed significantly from canola events GFP 3, GT 4, GT 8, and GT 9 (Fisher's PLSD, $P< 0.01$).

The seeds produced from *R. raphanistrum* GA maternal plants were germinated under greenhouse conditions (12 h day length, $22^{o}/26^{o}$ C night/day temperature). High rates of germination were observed, and no hybrids were detected based on the GFP phenotype among the 19,274 seedlings screened (Table 1).

Low *B. napus* to wild relative ratio (180:1)

In the low-density field experiment, dry environmental conditions after planting led to a slightly lower than expected germination rate of 7-8 canola plants per m^2 producing an average crop to wild relative ratio of 180 to 1. Both species initially flowered in early May, and flowering overlapped for over three weeks. Seeds were collected and cleaned from the *Br* CA plants in early June, and were stored at 4^o C for 3 months. After germination in soil, a total of 7,769 seedlings were screened for GFP fluorescence. The average hybridization frequency for all seedlings analyzed was 1.8% (range 0.08 ± 0.17% to 3.24 ± 7.73%)(Table 2). No significant differences in hybridization rates were detected with different transgenic *B. napus* events (ANOVA, $P > 0.05$).

Periphery experiment

A total of 11,392 seedlings were screened for GFP fluorescence, 6,870 seedlings from *Br* QC-2974 grown within the *B. napus* block and 4,522 seedlings from plants at the periphery of the block (Table 3). Within the *B. napus* block, *Br* QC-2974 plants exhibited a wide range of hybridization frequencies, ranging from 0.0% to 100.0%. The average hybridization frequency over all plants within the block was 37.2 ± 26.7%. No significant trends were detected between plants grown across the block (ANOVA, $P > 0.05$). The plants at the periphery demonstrated lower hybridization frequencies, averaging 5.2 ± 4.9% (range 0.0% to 17.1%). No significant differences were detected with distance (1, 2, 3 m) from the block (ANOVA, $P > 0.05$). The hybridization frequencies of plants grown within the block were significantly higher compared to those grown at the periphery (T-test, $P < 0.05$).

Backcrossing experiments

In the backcrossing experiment performed in North Carolina, several plots did not contain a transgenic GFP hybrid at the center of the plot, and were excluded from the study. Of the possible 51 plots, 16 plots contained a transgenic F_1 or BC_1 plant, and plots from the same *B. napus* event were combined for statistical analysis. The density of the surrounding *B. rapa* plants varied and ranged between 5-15 plants per 1 m^2 plot. From the maternal transgenic hybrid parents (F_1 and BC_1), a total of 2,095 seedlings were screened for GFP fluorescence, and 50,177 seedlings from maternal *B. rapa* plants were screened (Table 4). When the hybrid served as the maternal parent, backcrossing frequency ranged from 33.9 ± 16.3% with GT1 F_1 hybrids to 62.7 ± 13.5% with GFP1 F_1 hybrids. No significant differences were detected in the backcrossing frequency between canola events (ANOVA, $P > 0.05$), and the average for all hybrids was 44.8%. Backcrossing frequency on maternal *B. rapa* plants in each plot was significantly lower than the maternal hybrid plant, and ranged from an average of 0.026 ± 0.001% in F_1 GT5 plots to 0.076 ± 0.073% in F_1 GFP 1 plots. The average backcrossing frequency on *B. rapa* plants over all *B. napus* events was 0.088%.

In the Georgia location, 17 of the possible 30 plots contained a transgenic hybrid at the center of the plot. The density of the surrounding *B. rapa* plants varied and ranged between 5:1 and 15:1 plants per 1 m^2 plot. From the maternal transgenic hybrid parents, a total of 1950 seedlings were screened for GFP fluorescence, and 56,845 seedlings from maternal *B. rapa* plants were screened (Table 5). The F_1 or BC_1 transgenic hybrid as the maternal parent produced backcrossed seeds at frequencies ranging from 39.3% (BC_1 GT 8) to 56.0 ± 16.5% (F_1 GFP 2) with an average of 50% transgenic over all hybrids, and no significant differences were detected between canola events (ANOVA, $P > 0.05$). The

backcross frequency from transgenic hybrids to surrounding *B. rapa* plants within each $1m^2$ plot was low, ranging from 0% (BC_1 GT 8) to 0.12 ± 0.09 (F_1 GT 3) with an average backcrossing frequency of 0.060% over all seeds produced on *B. rapa* plants.

Discussion

From this report, transgene flow has been documented between ten independent transformation events of *B. napus* and *B. rapa* under field conditions. Several studies have shown that hybridization between canola and *B. rapa* (AA, 2n=20) occurs under field conditions and results in the production hybrid populations (Jorgensen and Anderson 1994, Landbo et al 1996, Hansen et al 2001, Halfhill et al. 2002, Warwick et al 2003). Hybridization occurs at a range of frequencies based on the ratio of parental crop species to the wild relative, and has been shown to be as high as 93% when single maternal *B. rapa* plants were widely dispersed in canola fields (Jorgensen and Anderson 1994). Transgenic canola varieties have been shown to hybridize with *B. rapa* under field conditions, and the frequency of hybridization was reported to range from 1% to 17% based on the transgenic variety (Halfhill et al. 2002). With relatively high hybridization frequencies, transgenic hybrids populations should be expected when these parental species occur in close proximity.

In the high *B. napus* to *B. rapa* ratio experiment, the variations in hybridization frequencies between canola events may be due to the transgene insertion locus, agronomic characteristics of the canola event, and differences within the field environment for that year. Future research will characterize the *B. napus* events under field conditions, in order to describe what factors led to differential hybridization rates. The low crop to wild relative ratio experiment illustrated that crop density may a play role in optimal hybridization

frequency, and lower hybridization frequencies with *B. rapa* may be seen when lower density stands occur due to volunteerism. Overall, hybridization between *B. napus* and *B. rapa* occurred over a wide range of experimental conditions.

Hybridization between GFP *B. napus* and *R. raphanistrum* was not detected in our study. Although several reports have documented the fact that this interspecific hybridization does occur (Eber et al. 1994, Baranger et al. 1995, Darmency et al. 1998, Chevre et al. 2000, Reiger et al. 2001), the frequency of hybridization was always rare. In Australia, gene flow between imidazolone-resitant *B. napus* and *R. raphanistrum* was detected at a low rate ($< 4 \times 10^{-8}$) and only when the *B. napus* served as the maternal parent (Reiger et al. 2001). In France, Chevre et al. (2000) estimated that the hybridization frequency was between 10^{-7} and 10^{-5}. In a recent report, Warwick et al. (2003) found a single herbicide (glyphosate) tolerant F_1 hybrid out of ca. 35,000 germinated seedlings. The utility of GFP for detecting very low frequency events (rarer than 1 in 10^6) is limited. Because the GFP system requires a visual assay of each individual for accurate screening, other systems, such as screening for resistance to an herbicide may be more effective when very large numbers of plants must be analyzed.

The backcrossing frequencies presented in this study are some of the first reported describing the transgene movement from a transgenic hybrid to wild relative. The low rates of backcrossing from transgenic hybrids to maternal *B. rapa* plants was unexpected. The expected rate of backcrossing was calculated to be 2.5% (1 of 40 seeds) based on the following factors. In our experimental design, the ratio of hybrid to wild relative was on average 1 to 10 (10% backcrossing expected initially). Warwick et al. (2003) demonstrated that hybrids have ca. 50% pollen fertility (10% divided by 2, resulting in 5%). Hybrid plants

were hemizygous for the transgene, and therefore 50% of its pollen should be transgenic (5% divided by 2, resulting in 2.5%). The experimentally determined backcrossing frequency of 0.074% (1 of 1,350 seeds) over both experiments was much lower than the expected rate. Future studies will be performed to characterize this phenomenon, and possible explanations include slight differences in flowering time, differential hybrid pollen fitness, and pollen competition between *B. rapa* and hybrid pollen at the level of the *B. rapa* stigma. Hauser et al. (1997) analyzed pollen competition between *B. napus* and *B. rapa* pollen on both maternal stigmas under greenhouse conditions, and found no significant differences between pollen types when *B. rapa* was the female parent. In contrast, our experiment showed that transgenic hybrids had low rates of successful pollination events on surrounding *B. rapa* plants under field conditions.

The backcrossing frequency onto the hybrid as the maternal parent was at the expected ca. 50% level. In this case, if the single transgenic hybrid was self-incompatible, all pollination should be from the surrounding *B. rapa* plants. The hybrid produces 50% transgenic ova, and therefore the expected number of transgenic progeny should be 50%. From these experiments, the transgenic hybrid has the greatest potential to generate progeny as the maternal parent, and transgenic hybrids should be the focus of regulatory strategies to manage transgenic populations under field conditions.

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Table 1. Field level hybridization with transgenic *Brassica napus* and wild relatives, *B. rapa* and *R. raphanistrum*, at a crop to wild relative ratio of 600 to 1. Number of seedlings screened and transgenics detected represent the sum all seeds analyzed from the wild relatives grown within a canola event, and the % hybridization represents the average hybridization frequency from wild relatives grown within a canola event. Total % hybridization represents the hybridization frequency of the summed totals. Different letters represent significant differences between hybridization frequencies (Fisher's PLSD, $P < 0.01$)

Brassica rapa

Canola event	No. of plants	No. of seedlings	No. transgenic	% hybridization
GFP 1	8	1108	161	12.39 ± 12.03 (ab)
GFP 2	8	1619	259	20.37 ± 19.82 (a)
GFP 3	8	1282	72	5.82 ± 5.30 (b)
GT 2	8	1585	297	17.11 ± 13.20 (ab)
GT 3	8	958	84	7.79 ± (ab)
GT 4	8	1731	85	4.80 ± 2.46 (b)
GT 5	4	162	44	22.02 ± 17.20 (a)
GT 6	4	897	67	7.47 ± 1.33 (ab)
GT 8	8	1733	60	3.97 ± 3.15 (b)
GT 9	8	1313	68	5.37 ± 6.63 (b)
totals	*72*	*12,388*	*1197*	*9.66*

Raphanus raphanistrum

Canola	No. of plants	No. of seedlings	No. transgenic	% hybridization
all events	*33*	*19,274*	*0*	*0.00*

Table 2. Field level hybridization with transgenic canola and a wild relative, *B. rapa*, at a crop to wild relative ratio of 180 to 1. Number of seedlings screened and transgenics detected represent the sum of all seeds analyzed from *B. rapa* CA plants isolated within a GFP canola event, and the % hybridization represents the average hybridization frequency of *B. rapa* plants within each canola event. Total % hybridization represents the hybridization frequency of the summed totals.

Canola event	No. of *B. rapa* plants	No. of seedlings	No. transgenic	% hybridization
GFP 1	14	3366	59	2.33 ± 3.06
GFP 2	13	3376	58	3.24 ± 7.73
GT 1	3	295	20	2.58 ± 4.48
GT 5	3	446	4	1.11 ± 0.98
GT 8	5	286	1	0.08 ± 0.17
totals	*38*	*7769*	*142*	*1.83*

Table 3. Hybridization frequency between *B. rapa* plants grown within and at the periphery of a GM *B. napus* field. Number of *B. rapa* plants represent the number grown at each location. Number of seedlings screened and transgenics detected represent the sum all seeds analyzed from the *B. rapa* plants grown at a location, and the % hybridization represents the average hybridization frequency from wild relatives grown at a location. Total % hybridization represents the hybridization frequency of the summed totals. Different letters represent significant differences between hybridization frequencies between plants within and the periphery of the field (T-test, $P < 0.05$).

Within field	No. of *B. rapa* plants	No. of seedlings	No. transgenic	% hybridization
field total	*102*	*6870*	*2681*	*37.2 ± 26.7 (a)*
Periphery	No. of *B. rapa* plants	No. of seedlings	No. transgenic	% hybridization
1 meter	10	1502	102	6.0 ± 4.0
2 meter	10	1555	106	5.8 ± 5.5
3 meter	9	1465	58	3.6 ± 5.1
periphery total	*29*	*4522*	*266*	*5.2 ± 4.9 (b)*
totals	***131***	***11,392***	***2947***	***25.9***

Table 4. Backcrossing frequency where a transgenic hybrid was surrounded by between 5-15 *B. rapa* plants in 1m^2 plots (Clayton, NC, USA). Seeds were collected from both the transgenic hybrid and each *B. rapa* plant within a plot. Number of seedlings screened and transgenics detected represent the sum all seeds analyzed from the hybrids or *B. rapa* plants from a hybrid plot, and the % hybridization represents the average hybridization frequency from hybrids and plots. Total % hybridization represents the hybridization frequency of the summed totals.

	Transgenic hybrid				***Brassica rapa***			
	No. plots	No. of seedlings	No. transgenic	% backcross	No. of plants	No. of seedlings	No. transgenic	% backcross
F_1 GFP 1	3	472	276	62.7 ± 13.5	24	9336	7	0.076 ± 0.073
F_1 GT 1	3	230	82	33.9 ± 16.3	18	8270	4	0.048 ± 0.014
BC_1 GT 1	1	187	91	48.7	8	3710	2	0.054
F_1 GT 4	3	645	302	47.6 ± 4.8	27	9994	12	0.125 ± 0.161
BC_1 GT 4	1	13	6	46.2	2	1488	3	0.202
F_1 GT 5	2	195	69	34.3 ± 34.8	17	7684	2	0.026 ± 0.001
F_1 GT 7	3	353	113	38.3 ± 26.2	22	9695	14	0.115 ± 0.081
totals	*16*	*2095*	*939*	*44.8*	*118*	*50,177*	*44*	*0.088*

Table 5. Backcrossing frequency where a transgenic hybrid was surrounded by between 5-15 *B. rapa* plants in 1m^2 plots (Tifton, GA, USA). Seeds were collected from both the transgenic hybrid and each *B. rapa* plant within a plot. Number of seedlings screened and transgenics detected represent the sum all seeds analyzed from the hybrids or *B. rapa* plants from a hybrid plot, and the % hybridization represents the average hybridization frequency from hybrids and plots. Total % hybridization represents the hybridization frequency of the summed totals.

	Transgenic hybrid				***Brassica rapa***			
	No. plots	No. of seedlings	No. transgenic	% backcross	No. of plants	No. of seedlings	No. transgenic	% backcross
F_1 GFP 2	4	330	191	56.0 ± 16.5	45	13,037	4	0.039 ± 0.060
F_1 GT 3	5	1064	520	49.8 ± 9.0	45	19,470	22	0.121 ± 0.091
BC_1 GT 3	3	186	85	46.0 ± 6.9	32	9106	2	0.024 ± 0.041
F_1 GT 8	4	342	176	51.0 ± 2.1	51	11,832	6	0.057 ± 0.071
BC_1 GT 8	1	28	11	39.3	9	3400	0	0.000
totals	*17*	*1950*	*983*	*50.4*	*182*	*56845*	*34*	*0.060*