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I am submitting herewith a thesis written by Hong Seok Moon entitled "Risk assessment of genetically modified crops by direct tracking pollen movement and testing crop genetic load using directly transformed *Brassica rapa* with Bt *cry1Ac* and *gfp* genes." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Plant Science.

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Risk assessment of genetically modified crops by direct tracking pollen movement and testing crop genetic load using directly transformed *Brassica rapa* with Bt *cry1Ac* and *gfp* genes

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Abstract

One concern with crop biotechnology is that there might be crop to weed transgene flow, which could result in more invasive and competitive weed populations. Transgene expression, introgression of crop genes, and other ecological factors may alter the fitness or productivity of weed populations. The *Brassica napus* (crop) to *Brassica rapa* (weed) model to assess transgene flow and consequences has been widely used. In this study, weedy accessions of *Brassica rapa* were transformed with *Bacillus thuringiensis* (Bt) *cry1Ac*- and green fluorescence protein (GFP)- coding transgenes using *Agrobacterium* to develop plants to be subsequently used in risk assessment research. Regenerated transgenic *B. rapa* lines were characterized by progeny analyses, Bt protein enzyme-linked immunosorbent assay (ELISA), Southern blot analysis, and GFP expression assays. GFP expression level and Bt protein concentration were significantly different among independently transgenic *B. rapa* events. Seed yield of transgenic *B. rapa* events was compared to *B. rapa* × *B. napus* introgressed hybrids in greenhouse and field experiments as comparative tools to evaluate the genetic load of introgressed crop genes in weedy populations. In a greenhouse study, the biotypes expressing the Bt transgene were significantly different from insect susceptible plants and insect resistance was the predominant factor in productivity under diamondback moth (*Plutella xylostella*) herbivore pressure. No significant differences were observed, however, in vegetative growth or reproductive yield between the transgenic *B. rapa* lines and crop-weed hybrids under field conditions. Directly transformed transgenic *B. rapa* plants were an essential

positive experimental control to begin to assess genetic load of crop genes in crop-weed hybrid populations. This is the first report of the direct transformation of a weedy plant.

Transgene movement via pollen is an important parameter for understanding and evaluating possible out-crossing capacities of transgenic crop varieties. Here, we describe the movement of oilseed rape (*Brassica napus* L. cv. Westar) pollen expressing a genetically encoded fluorescent tag. Transgenic oilseed rape plants were produced using *Agrobacterium*-mediated transformation method with the pBINDC1 construct containing a GFP variant, *mGFP5-ER*, under the control of the pollen-specific LAT59 promoter. Transgenic pollen was differentiated from non-transgenic pollen *in vivo* by a unique spectral signature and was shown to be an effective tool to monitor pollen movement in proof-of-concept studies in the greenhouse and field. GFP-tagged pollen also served as a practical marker to determine the zygosity of plants. In a greenhouse study, more pollen was captured at closer distances from the source plant plot with consistent wind generated by fans. Under field conditions, GFP transgenic pollen grains were detected up to 15 meters from the source plants. No significant difference was detected under field conditions for pollen frequency among distances 0, 5, 10, and 15 m from the source plant plot with no consistent wind effects on the number of pollen grains detected on pollen traps. No significant differences between transgenic pollen and non-transgenic pollen were detected for pollen dispersal under field conditions.

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Chapter one

Literature Review

Risk assessment of genetically modified crops

The global transgenic crop acreage has increased yearly since the commercial introduction of agronomically-improved transgenic crops in 1996. The majority of transgenic crops were grown in the US, Argentina and Canada, and many countries are in the process of assessing the risks of transgenic crops and considering legislation to regulate the use of commercially available transgenic crops (Dunfield and Germida 2003). A debated environmental concern posed by transgenic crops is transgene escape from transgenic crops to wild relatives or feral populations (Stewart et al. 2003). Transgenic crops engineered with specialized traits such as insect resistance could transfer novel genes to nearby weeds through hybridization (Eber et al. 1994; Jorgensen and Andersen 1994; Chevre et al. 1997). Further, seed dispersal and plant persistence are other factors researched to cause movement of transgenes, and thus, should be evaluated for potential ecological risk (Crawley and Brown 1995; Crawley et al. 1993; Hails 2000).

Oilseed rape as a model plant for risk assessment

Oilseed rape (*Brassica napus* L. AACC 2n=38) is now one of the largest oilseed crops in the world and widely used as a cooking oil, salad oil, and margarine ingredient. Oilseed rape is an appropriate crop for the study of transgene escape or movement, since oilseed rape has many wild relatives such as field mustard (*Brassica rapa* L.AA 2n=20) and wild radish (*Raphanus raphanistrum*) which occur as weed populations in or near oilseed rape cultivation areas and overlap the flowering period of oilseed rape (Simard et al. 2002; Halfhill et al. 2002; Chevre et al. 2003). Unlike oilseed rape, crops such as corn, potato and cotton cannot be pollinated with their feral species (Mendelsohn et al. 2003). Oilseed rape is categorized as a moderate risk crop for introgression between crops and wild relatives (Stewart et al. 2003). Oilseed rape pollen can move up to 3 kilometers between fields in the air (Stokstad 2002). Oilseed rape is a partially self-fertilized species, and self-fertilization rate has ranged from 53-88% depending on cultivar and environmental conditions (Becker et al. 1992). Oilseed rape has been shown to persist at least 8 years outside of cultivated fields (Pessel et al. 2001). Transgenes for herbicide or insect resistance likely transferred to nearby weeds, creating weeds resistant to insects or herbicides and thus, could be more difficult to control. Oilseed rape and field mustard are known to hybridize (Mikkelsen et al. 1996), and field mustard is the wild relative that most easily hybridizes with oilseed rape.

Herbicide resistance has been engineered into *B. napus* and grown commercially, and insect resistance has been engineered in for experimental purposes with prospects for commercial release. The transfer of herbicide tolerance genes from commercial oilseed rape to naturally occurring wild relative populations was reported in Quebec, Canada via

hybridization (Warwick et al. 2003). Transgene flow from transgenic oilseed rape to wild relatives for insect resistance has been shown to occur under varied field conditions (Halfhill et al. 2004). Genes transferred from transgenic crops to their weedy relatives could result in the creation of transgenic hybrid weeds; there is no doubt that this is the case with *B. napus* × *B. rapa*. Therefore, quantifying the fitness of transgenic hybrid weeds is important for evaluating the potential risk of gene flow from transgenic crops to closely related weed species, i.e. determining the consequences of gene flow.

The fitness of some crop-weed hybrids has been shown to be relatively higher when compared to their parents (Klinger and Ellstrand 1994; Hauser et al 1998a,b). In contrast, some researchers have shown that crop-weed hybrids had lower fitness than wild genotypes (Snow et al. 1998; Halfhill et al. 2005). Determination of whether transgenic hybrid weeds have higher fitness than non-transgenic weeds under field conditions will be beneficial in the understanding of the potential risk of gene flow from transgenic crops to weeds.

Green fluorescent protein (GFP) as an *in vivo* marker for gene flow

The potential escape of transgenes could result in fitness-enhanced weedy relatives (Klinger and Ellstrand 1994; Hauser et al. 1998a) that would warrant the need for an *in vivo* gene monitoring system to quantify and assess ecological risks in the field. An important tool for monitoring possible introgression of genes such as herbicide, disease, insect, and drought resistance into weedy relatives is green fluorescent protein (GFP) tagging (Stewart 1996, 2005). GFP was isolated and cloned in 1992 from a jellyfish,

Aequorea victoria. GFP consists of 238 amino acids with wild type excitation peaks of 395nm and 475nm and an emission peak of 508nm (Chalfie et al. 1992). Several variants with increased fluorescence level and improved expression in eukaryotic cells have been developed by modifying codon usage or adding peptide targeting sequence (Haseloff et al. 1997; Siemering et al. 1996). GFP is a valuable tool used to assess the frequency of stable transformation during tissue culture as well as to monitor the gene flow from transgenic plants in the environment (Stewart 1996; Harper et al. 1999). GFP does not require a co-factor for fluorescence, which makes it an effective, noninvasive, *in vivo* marker for gene expression (Leffel et al. 1997). GFP had a low risk of allergenicity in an orally administered experiment with rats and is not likely to cause a health risk (Richards et al. 2003). GFP variant *mGFP5-ER* is a good variant as a visual marker to detect plants that express GFP in pollen or throughout the whole plant under the control of the CaMV 35S promoter (Halfhill et al. 2001). GFP also has been shown to serve as a vital screenable marker in rice transformation (Vain et al. 1998). The *mGFP5-ER* is an appropriate variant for this study since it has been shown to work well under field conditions and is strongly visible in plant cells (Harper et al. 1999, Stewart 2001). The inheritance and expression stability of foreign genes has been studied in different transgenic plants (Duan et al. 1996). The expression of GFP and its inheritance were studied in transgenic oat (*Avena sativa* L.) plants transformed with a synthetic GFP gene [*sgfp*(S65T)] driven by a rice actin promoter (Cho et al. 2003). GFP expression was stably inherited in some plants, but some plants had transgene silencing – the gene was present as determined by PCR but expression of the transgene was not observed (Cho et al. 2003). GFP has become an invaluable tool in plant research and it has been to be an

effective tool to monitor the expression and possible introgression of transgenes from crops into their wild relative species (Stewart 1996; 2005; Halfhill et al. 2001; 2003a). GFP may be a suitable marker for the direct detection of pollen-mediated gene flow (Hudson et al. 2001).

Transgene flow via pollen movement

Transgene movement via pollen flow is a prominent mode for transferring transgenes in the environment. The use of transgenic plants has proven to be an effective tool to quantify gene flow (Messeguer 2003). Gene flow is likely to occur via pollen movement since reproductive organs are intended to create gene movement (Saeglitz et al. 2000). Transgene movement occurs by pollen dispersal into their wild relatives, potentially resulting in enhanced weed populations (Scheffler and Dale 1994). Gene flow through pollen has been demonstrated in sugar beet, oak, rice, oilseed rape, and barley (Alibert et al. 2005; Dutech et al. 2005; Rieger et al. 2002; Damgaard and Kjellsson 2005; Chen et al. 2004; Song et al. 2004; Ritala et al. 2002). Oilseed rape and weedy *B. rapa* are able to hybridize and backcross spontaneously in both experimental plots and cultivated fields (Mikkelsen et al. 1996; Wilkinson et al. 2000). Approximately half of the transgenic oilseed rape pollen, which is heavy and sticky, fell onto plant surfaces and the ground within 3 m, but a small percentage became airborne in the wind (Lavigne et al. 1998). In plants, gene flow can occur through seed or pollen dispersal. Gene flow via pollen dispersal was found to be greater than would be inferred from pollinator

movement alone (Schaal 1980). However, pollen dispersal by either wind or pollinator is an important mode of transgene escape or movement.

Chapter Two

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

* This chapter has been submitted to Plant Cell Reports with authors Hong S. Moon, Matthew D. Halfhill, Laura L. Good, Paul L. Raymer, and C. Neal Stewart, Jr.

Abstract

Crop to weed transgene flow, which could result in more competitive weed populations, is a transgenic plant biosafety concern. Transgene expression, introgression of crop genes, and other ecological factors may alter the productivity of weed populations. Directly transformed weeds are apt comparisons for introgressed transgenic hybrids. Weedy *Brassica rapa* accessions were transformed with *Bacillus thuringiensis* (Bt) *cry1Ac* and green fluorescence protein (GFP) transgenes using *Agrobacterium* to produce plants to be used in risk assessment research. Regenerated *B. rapa* events were characterized by Southern blot, Bt protein ELISA, and GFP expression assays. Productivity of transgenic *B. rapa* events was compared to *Brassica* crop-weed introgressed hybrids in greenhouse and field experiments. GFP expression level and Bt

protein concentration were significantly different among independently transgenic *B. rapa* events. In the greenhouse study, insect resistance was the predominant factor in productivity under diamondback moth (*Plutella xylostella*) pressure. This is the first report of the direct transformation of a weedy plant.

Introduction

While transgenic plants have been grown commercially for over ten years, there is continued debate about their risks and regulation (Stewart 2004). Some level of gene movement in commercial transgenic crop field is likely to be inevitable (Timmons et al. 1996). However, the risk of transgene movement from oilseed rape (*Brassica napus*, OSR) to its relatives is quite low, with the exception of gene flow to *Brassica rapa*, where interspecific hybridization occurs readily (Legere 2005). It has long been thought that hybridization of transgenic crops with other crop cultivars or with related weed species could result in the creation of more competitive and invasive hybrid populations (Mikkelsen et al. 1996; Stewart et al. 2003; Al-Ahmad et al. 2004).

Hybridization between transgenic OSR and wild relatives has been confirmed by the presence of crop-specific markers and transgenes in hybrid populations (Legere 2005). The transfer of an herbicide (glyphosate) tolerance gene from commercially cultivated fields of OSR to a naturally occurring wild population of *B. rapa* producing F_1 hybrids was reported in Quebec, Canada (Warwick et al. 2003). In field experiments, the flow of an insect resistant transgene such as *Bacillus thurigiensis* (Bt) *cry1Ac* from transgenic OSR to wild relatives occurred under varied field conditions (Halfhill et al. 2004). The

fitness of some *Brassica* crop-weed hybrids has been shown to be relatively high compared to their parents (Klinger and Ellstrand 1994; Hauser et al. 1998a,b). In contrast, crop-weed hybrids have had lower fitness than wild genotypes of their parents (Snow et al. 1998; Halfhill et al. 2005). Lower fitness of hybrid plants may be caused by the presence and expression of transgenes, introgressed crop genes, or other ecological factors.

To date, many researchers have attempted to determine the consequences of hybridization and introgression between transgenic crops and their wild relatives (Snow and Moran-Palma 1997; Gueritaine et al. 2002; Chevre et al. 2003; Halfhill et al. 2005; Legere 2005), but little is known about the persistence or ecological effects of crop genes that enter wild populations through pollen movement (Snow and Moran-Palma 1997). Halfhill et al. (2005) have reported that crop-weed hybrids, with or without transgene introgression, had lower fitness and competitive ability than their parent populations suggesting that the fitness depression of crop-weed hybrids may be caused by crop genes in hybrids or other factors rather than the presence of the transgene itself. The expression of Bt *cry1Ac* transgenes have no fitness penalty in transgenic OSR (Mason et al. 2003), and GFP is also an ecologically neutral transgene (Harper et al. 1999; Stewart 2006). Changes in fitness of crop-weed hybrids could be caused by a transgene spread in association with other crop genes during the hybridization and introgression process (Landbo and Jorgensen 1997). In some crop species such as sugar beet, the genetic background was found to be much more important than the presence of transgenes for fitness or productivity (Crawley et al. 2000).

OSR is an important oilseed crop worldwide with transgenic OSR encompassing a large percentage grown of commercially-grown crop (Warwick et al. 2003). OSR is cultivated in close proximity to and shares an overlapping flowering period with wild relatives, making oilseed rape an appropriate crop for the study of transgene escape (Holm et al. 1997; Chevre et al. 2003). *B. rapa* is the genetically closest wild or weedy related species with high risk of gene flow from cultivated oilseed rape plants (Legere 2005). Transgenic insecticidal OSR carrying the Bt transgene has shown increased fitness under insect selection conditions (Stewart et al. 1997). Transgene flow from Bt transgenic OSR to its wild relatives via hybridization and backcrossing has been reported in experimental field conditions using GFP expression as a marker in plants (Halfhill et al. 2002).

The purpose of our study was several-fold. First, in order to control for genetic background to assess the effect of introgression to weedy *B. rapa* in subsequent experiments, we produced directly transgenic *B. rapa* events with Bt *cry1Ac* and *gfp* transgenes via an *Agrobacterium*-mediated transformation method. A directly transformed *B. rapa* is an essential positive experimental control to use as a transgenic weed that would not carry any crop-specific genes which could be introgressed in crop-weed hybrids. Second, we characterized the transgenic events using molecular methods. Third, this study also aimed to begin to test the effect of crop genetic load in introgressed crop-weed hybrids by comparing these to transgenic *B. rapa* lines under greenhouse and field conditions.

Materials and Methods

A synopsis of *Brassica* plant types used in this study are listed in Table 1. Essentially we used a oilseed crop-type *Brassica napus* cv. 'Westar' along with transgenic derivatives and weedy *Brassica rapa* along with transgenic derivatives and backcrossed hybrids between the two species.

Plant transformation

Plant accessions

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

Vectors

Two gene constructs were used for plant transformation, including pBin-mGFP5-ER (GFP gene only) and pSAM12 (*mGFP5-ER*-Bt *Cry1Ac*; described in Harper *et al*., 1999). The pSAM12 construct contains *mGFP5-ER*, synthetic Bt C*ry1Ac*, and kanamycin resistant *nptII* genes carried in the T-DNA, enabling all three traits to be inserted into a single, genetically linked locus. The Bt and GFP transgenes were expressed under the control of the separate CaMV 35S promoter in separate cassettes on a single T-DNA vector.

Table 1 List of plant biotypes used in this study. Three weedy *B. rapa* accessions and one *B. napus* cultivar were used as parents. Two different constructs (pSAM12 contains GFP/Bt genes and pBIN-mGFP5-ER contains GFP gene) were used. All are under the control of the 35S promoter.

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

Weedy B. rapa transformation

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

Southern blot analysis

A southern blot analysis was performed to confirm transgene presence in directly transformed *B. rapa*. Genomic DNA was extracted from frozen leaf tissue of GFP transgenic *B. rapa* events and wild QC2974 *B. rapa* using CTAB (Dellaporta et al. 1983). After digestion of 10 µg of genomic DNA with *Hin*dIII, fragments were purified with QIAquick PCR purification columns (QIAGEN, Valencia, CA, USA). Control plasmid DNA from the binary vector pBIN-mGFP5-ER was also digested with *Hin*dIII. The

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

GFP fluorescence detection and analysis

Quantification of GFP fluorescence was performed using a Fluoromax -2 fluorescence spectrophotometer (Instruments S.A., Edison, NJ, USA) utilizing *DataMax* software (Galactic Industries Corporation, Salem, NH, USA). A 2 m bifurcated fiber cable was used to transmit excitation light and detect emission transmission from the leaves. Fluorescence spectrometry was performed in the middle of third leaf at four-leaf stage of GFP *B. rapa* events (transformed *B. rapa* with GFP gene), GT *B. rapa* events (transformed *B. rapa* with GFP/Bt genes), Bt BC_2F_2 hybrid, and wild *B. rapa* QC2974 accession plants. All plants were excited with UV light (385 nm) and scanned from 440

to 600 nm. For this study, the 450 nm wavelength served as the anchor point (Millwood et al. 2003). GFP fluorescence of the transgenic *B. rapa* events at 508 nm was subtracted from the anchor point average value for multiple measurement of wild QC 2974 *B. rapa* plants.

Bt expression analysis

Expression of Cry1Ac protein in the transgenic *B. rapa* events was quantified by Bt enzyme-linked immunosorbent assay (ELISA) using a Qualiplate^{TM} kit for Cry1Ab/Cry1Ac (Envirologix Inc, Portland, ME, USA). Total soluble protein was extracted from leaves using the protein extraction method described in Stewart et al. (1996). Fresh leaf tissue (0.2 g) from the transgenic *B. rapa* events and Bt BC_2F_2 (transgenic backcrossed generation between BnGT and QC2974; described in Halfhill et al. 2005) was collected and homogenized in 400 µl of 0.1 N NaOH using a power drill in a microcentrifuge tube. The samples were incubated on ice for 30 min, and 80 µl of 1 M Tris-HCl (pH 4.5) was then added for neutralization. Each sample was clarified by centrifugation at 8,765 ×g for 5 min. The supernatant was discarded and the remaining fraction was quantified by a Bradford assay. Total soluble protein per sample was quantified by Coomassie Plus™ Bradford Assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Soluble protein from each sample was diluted to 50 µg of protein per ml and 5 µg of soluble protein was put into each respective sample well. Qunatification of Bt protein by ELISA was then performed using a Qualiplate™ kit for Cry1Ab/Cry1Ac according to manufacturer's instructions.

Transgenic B. rapa plants under insect pressure in greenhouse

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

Plant types

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

Plot design and application of insects

One hundred fifty 4 L pots were arranged based on a randomized complete block design (RCBD) and divided into three replicates. Thirty pots were assigned to each block. Five pots in each block were designated to each plant type. Randomly half of six blocks were under herbivory pressure. A strain of diamondback moth (DBM) (*Plutella xylostella*) (Benzon Research Inc. Charlisle, PA, USA) susceptible to Bt was used as the herbivore. At 8-12 leaf stage of plant, 10 neonate diamondback moths at the 3rd instar were applied

to each plant using a small paint brush. After 2 weeks, the insecticide Marathon® 1% Granular (Olympic horticultural products Co., Mainland, PA, USA) was applied to all plants in order to prevent immigration of DBM to other blocks. Because of the toxicity of insecticide to DBM, no more damage was expected by DBM after insecticide application on both treatment blocks.

Quantifying herbivory damage

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

Harvesting and analyses

At maturity, plant above-ground biomass was harvested and each plant was stored in a separate mesh bag for a month at 36±2ºC until the plants were completely dried. Dry weight of each individual plant was recorded and then seeds were cleaned. The number of total seeds per plant was calculated based on average weight of 100 seeds. Plant vegetative and reproductive productivity were estimated by plant dry weight and number of seeds, respectively. Plant productivity data for insect applied blocks and no insect blocks were analyzed by analysis of variance (ANOVA) using SAS version 9.1. Differences of productivity among plant types were also analyzed.

Productivity of transgenic B. rapa events under field conditions

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

Plant types

Ten types of plants were used in this study. Wild *B. rapa* (QC2974), 3 events of transgenic GFP *B. rapa* (74-GFP2, 74-GFP3, and 74-GFP5), 2 events of transgenic GT *B. rapa* (74-GT1 and 74-GT2), Bt BC_2F_2, and BC_2F_2 crop-weed hybrids, homozygous transgenic *B. napus* for GFP/Bt genes (BnGT1; described in Halfhill et al. 2001), and *B. napus* (cv. Westar) (Table 1). All transgenic *B. rapa* events and Bt BC_2F_2 and were screened with a hand-held UV light in the dark to confirm expression of the GFP transgene.

Plot design

The field experiment was designed based on a RCBD with 8 replicates. A total of 80 plots were sown (10 plant types × 8 replicates). Each plot size was 1 m^2 and the isolation between plots was 1 m. Seeds were scattered by hand into each respective plot at a density of 200 seeds per m^2. After 72 days post seed sowing, all GFP and GT events of transgenic *B. rapa* plants and Bt BC_2F_2 were screened with a hand-held UV light. The plant number in each plot was thinned to 15 plants.

Harvesting and statistical analyses

At maturity, the above-ground vegetative biomass from each plot was harvested separately using a line trimmer (Weed Eater 22" Excalibur, Electrolux Group, Nashville, AR, USA). Plant productivity was measured and the data were analyzed as described previously for the greenhouse experiment. Differences of productivity among plant types were compared by ANOVA and contrasts using SAS version 9.1.

Results

Weedy B. rapa transformation and regeneration

Fifteen independent transgenic *B. rapa* events were generated from three weedy *B. rapa* accessions (QC2974, QC2975 and CA). Callus was induced from the ends of the chopped hypocotyl segments within 14 days on callus induction medium. Transformed callus sectors fluoresced green under UV light. The pBIN-mGFP5-ER and pSAM12 constructs generated 21 and 14 independent fluorescent sectors, respectively (Table 2). Roots formed on shoots from all accessions with both constructs. Wild weedy *B. rapa* QC2974 transformed with the pBIN-mGFP5-ER construct had relatively higher transformation efficiency calculated based on the number of fertile plants out of total explants. Transformed plants were confirmed by GFP screening under UV light and were easily distinguished from wild *B. rapa* plants (Fig. 1). T_1 seeds from each transgenic event were acquired from a hybrid cross between T_0 transgenic *B. rapa* plants and the respective wild *B. rapa* accession via hand-crossing. Each T_1 transgenic *B. rapa* event segregated for GFP expression, except 74-GFP5 event which was all positive for

Table 2 Summary of weedy *Brassica rapa* accessions transformation efficiency using *Agrobacterium*-mediated method. Three weedy accessions of *B. rapa* were transformed with two different constructs including pBIN-mGFP5-ER and pSAM12. The pBIN-mGFP5-ER contains GFP gene and the pSAM12 contains GFP/Bt genes; all are under the control of the 35S promoter.

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

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

[b] Number of GFP fluoresced sectors

[c] Number of shoots regenerated

[d] Number of shoots that formed roots

[e] Number of transgenic plants that produced T_1 seeds

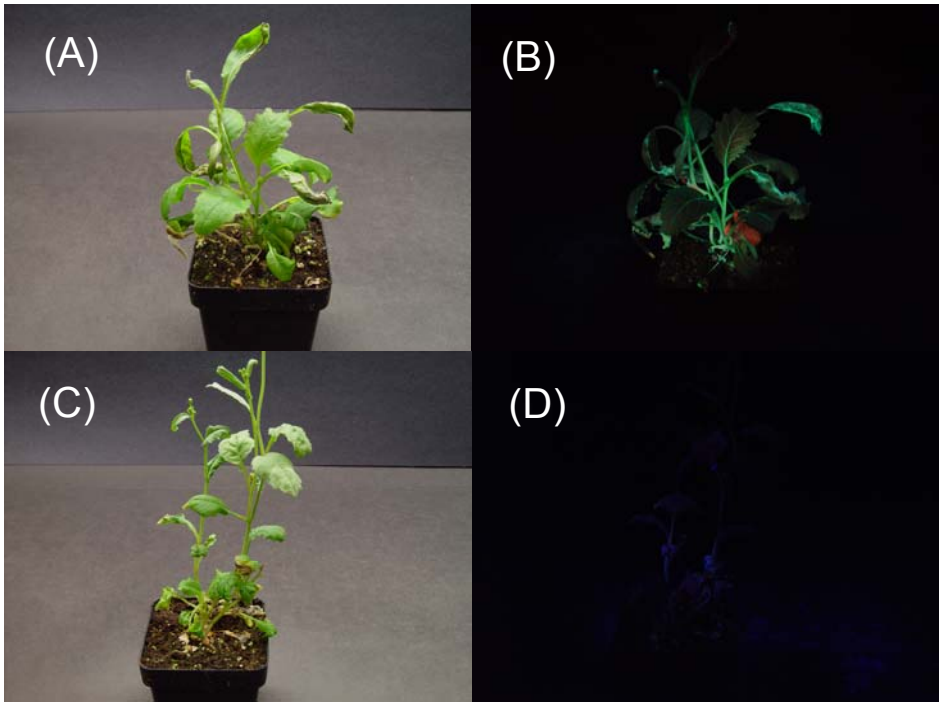


Figure 1 Detectable GFP fluorescence in transformed *B. rapa* plants. QC2975 plant transformed with *Agrobacterium* containing *mGFP5-ER* was transferred to soil and photographed under normal light (A), under UV light (B). Non-transgenic QC2975 plant was photographed under normal light (C), under UV light in the dark (D).

GFP expression (data not shown). Plants within each transgenic event were crossed among GFP positive T_1 transgenic plants from independent event to acquire T_2 seeds. Only 9 transgenic *B. rapa* events out of total 15 established events were used for the analysis or characterization.

Number of transgenes integrated in transgenic B. rapa

Independent T_2 GFP transenic *B. rapa* events were analyzed through Southern blot analysis using *Hin*dIII digested genomic DNA (Fig. 2). Since *Hin*dIII restricts the T-DNA insert before 5' to the *mGFP5-ER* gene, probing with GFP yields a single hybridizing band for each T-DNA insert. Southern blot analysis results confirmed multiple transgene integrations in several events, including 74-GFP2, with two insertions, and 74-GFP5 and CA-GFP1, which both appear to contain four T-DNA inserts. The hybridizing bands in genomic digests varied in size between transgenic events and were not identical to hybridizing bands in *Hin*dIII digested binary vector control, indicating the GFP transformants were independent and transgenes were stably integrated in the *B. rapa* genome.

GFP fluorescence of transgenic B. rapa events

GFP expressions from transgenic *B. rapa* T_2 plants of each GFP and GT events were measured using a fluorescence spetrophotometry. There were significant differences for emission intensity at 508 nm among transgenic *B. rapa* events and hybrid Bt BC_2F_2 (ANOVA, $P<0.05$) (Fig. 3). The CA-GFP1 event exhibited the highest average 508 nm emission peaks at 3.6±0.3 cps (10^5) (Fig. 3). Transgenic *B. rapa* plants and Bt BC_2F_2 had

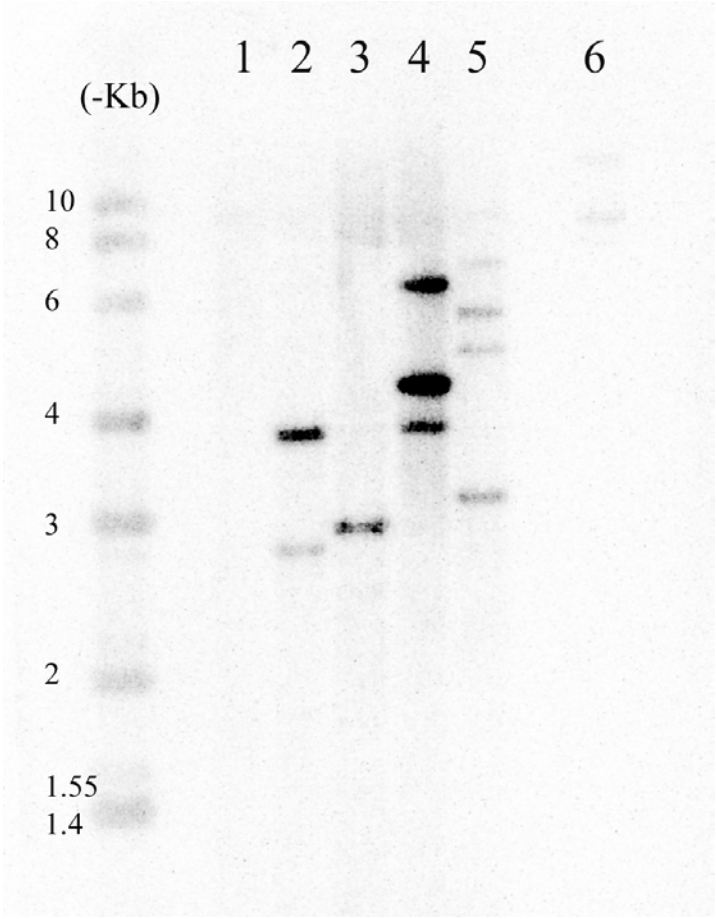


Figure 2 Southern blot analysis of T_2 GFP *B. rapa* events. Southern blot analysis of *Hin*dIII digested genomic DNA hybridized to GFP probe. Genomic DNA from untransformed *B. rapa* QC2974 (lane 1), three independent GFP transgenic events of *B. rapa* QC2974 (74-GFP2, 74-GFP3, 74-GFP5, lanes 2-4),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

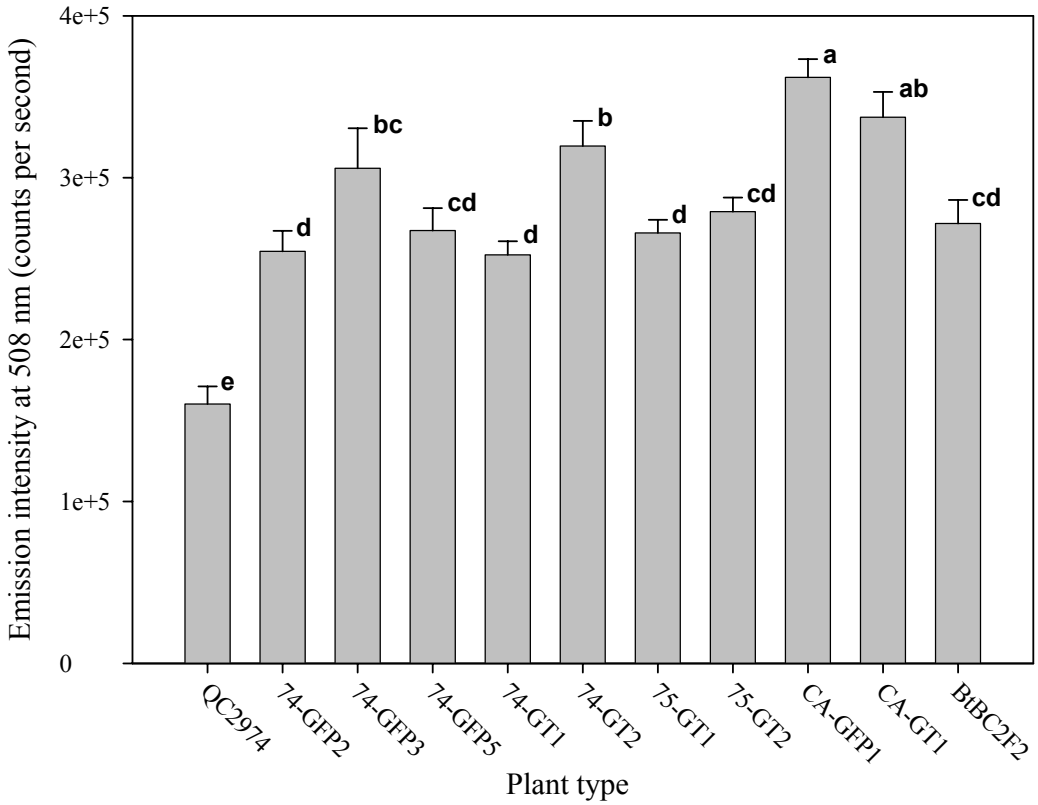


Figure 3 Fluorescence average at 508 nm for T_2 9 independent transgenic *B. rapa* events and Bt BC_2F_2 hybrid. Wild weedy accession (QC2974) *B. rapa* served as a experimental control. Emission intensity was recorded in counts per second (10^5). Different letters represent significant difference between plant types (ANOVA, $P<0.05$).

a GFP expression at 508 nm from 2.5±0.4 to 3.6±0.3 cps (10^5) (all units in 10^5 counts per second).

Bt cry1Ac protein quantification

Transgenic GT *B. rapa* events and Bt BC_2F_2 hybrid expressed Bt Cry1Ac protein at varying levels from 0.016 to 0.045% of total soluble protein (Fig. 4). As expected, no Bt protein was detected from wild *B. rapa* and transgenic GFP *B. rapa* events. Significantly different Bt concentrations were shown among independent GT *B. rapa* events (ANOVA, $P<0.05$). Bt BC_2F_2 hybrid expressed less Bt protein than transgenic GT *B. rapa* events (ANOVA, $P<0.05$).

Productivity of transgenic B. rapa events under insect pressure

Estimated herbivory damage by visual assay varied between types of plants (Fig. 5). Little herbivory damage was observed on the plants containing Bt transgene, including 74-GT2 and Bt BC_2F_2 plants; however non-Bt plants QC2974, 74-GFP3, and BC_2F_2 had significant herbivory damages on leaves (ANOVA, $P<0.05$). Herbivory damage was observed only in insect applied blocks.

When the comparison of vegetative productivity was made within a single biotype plant, most biotypes had similar vegetative weight in the presence or absence of insect pressure (Fig. 6). Significant difference was detected for vegetative productivity among different plant types (ANOVA, $P<0.05$). Reproductive productivity within a single plant type was significantly different between insect and no insect pressure (ANOVA, $P<0.05$) (Fig. 6). Wild *B. rapa* and BC_2F_2 plants grown under herbivory pressure had a lower

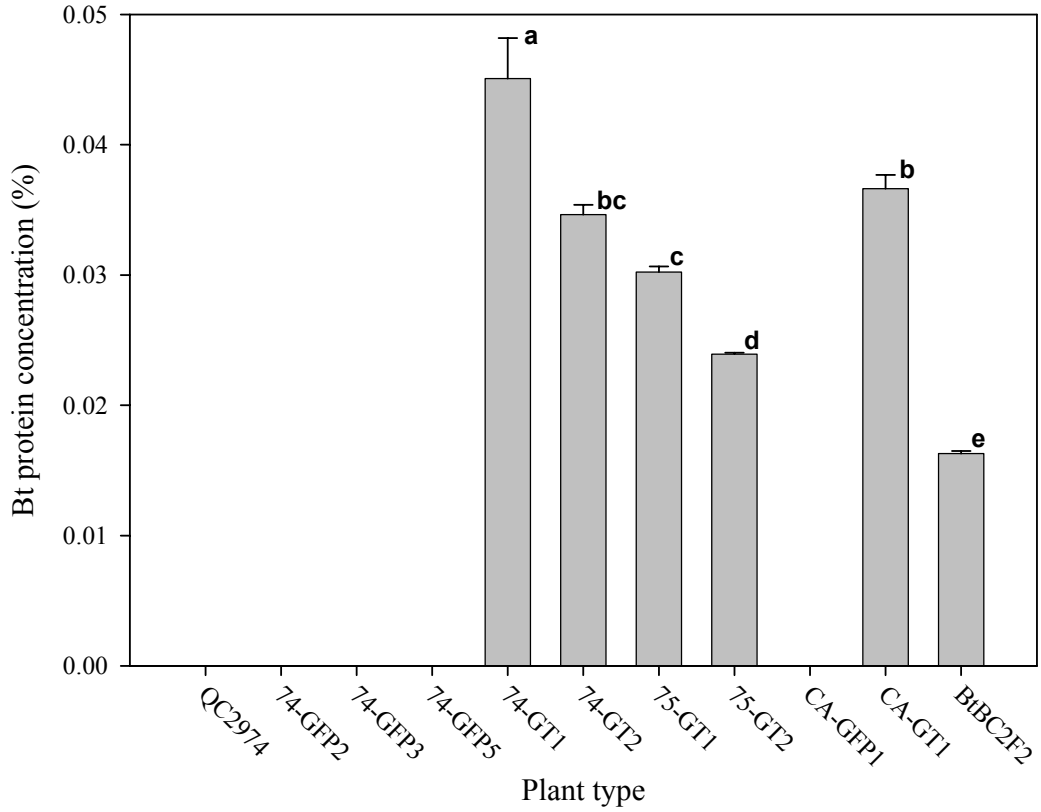


Figure 4 Bt *cry1Ac* protein concentration of transgenic *B. rapa* events and Bt BC_2F_2 hybrid from enzyme-linked immunosorbent assay (ELISA). Different letters represent significant differences between independent transgenic lines (ANOVA, $P<0.05$). Error bars represent ± standard error of the mean.

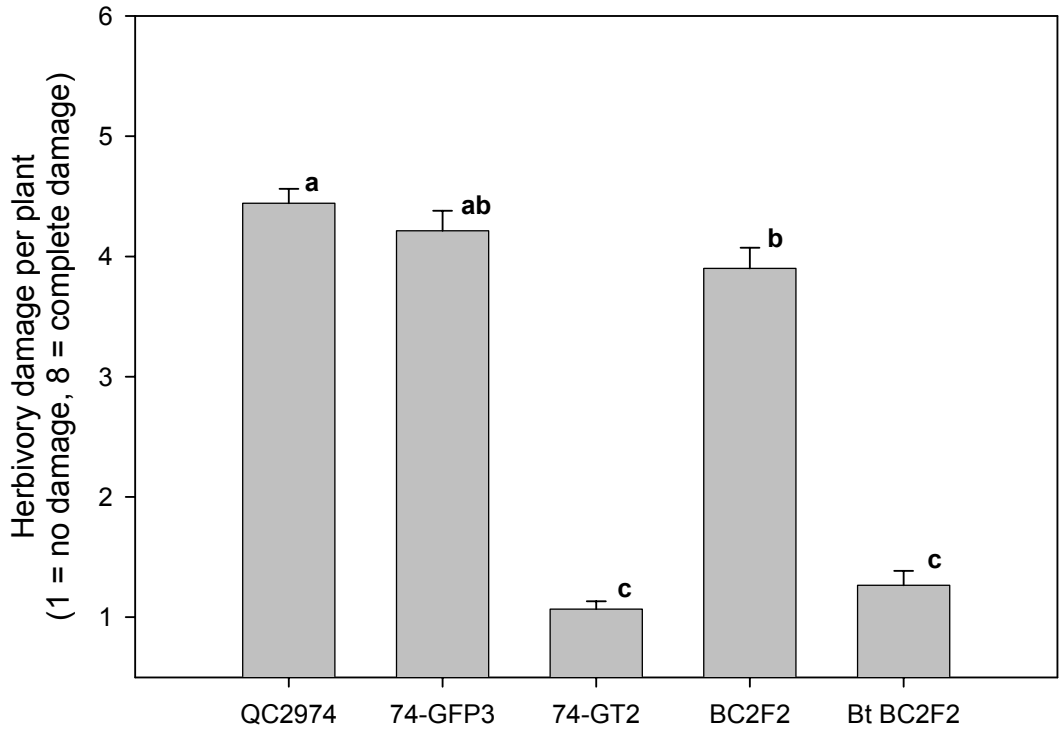


Figure 5 Herbivory damage of plants used in greenhouse study under diamondback moth (DBM) pressure. Different letters represent significant differences between plant types (ANOVA, $P<0.05$). Error bars represent ± standard error of the mean.

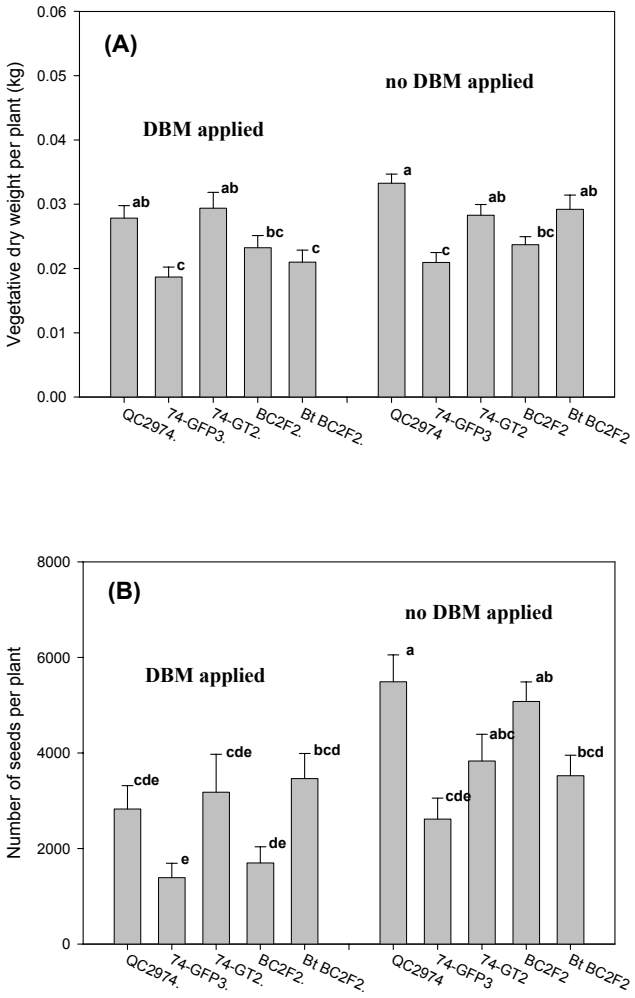


Figure 6 Productivity of transgenic *B. rapa* events in greenhouse with diamondback moth (DBM) pressure. Five plant types were planted and grown including wild QC2974, 74-GFP3, 74-GT2, BC_2F_2, and Bt BC_2F_2 hybrids. Panel (A) represents vegetative productivity, and panel (B) represents reproductive productivity. Different letters represent significant differences between treatments (ANOVA, $P<0.05$). Error bars represent ± standard error of the mean.

reproductive productivity (ANOVA, $P<0.05$) than those grown under no insect pressure, indicating that hybrid status was the main factor in decreased productivity (Halfhill et al. 2005). Wild QC2974 produced significantly more seeds than Bt BC_2F_2 under no insect pressure; however, wild QC2974 had similar vegetative weight with Bt BC_2F_2 under the same condition.

Transgenic B. rapa plants productivity under field conditions

Significant differences for vegetative productivity among different plant types under field conditions were observed (ANOVA, $P<0.05$) (Fig. 7). As expected, Westar and BnGT1 had significantly higher plant dry weight than all *B. rapa* plants and crop-weed hybrids (ANOVA, $P<0.05$). No significant differences for vegetative productivity between wild QC2974 and transgenic *B. rapa* events were evident (Contrast, $P=0.53$). Both Bt BC_2F_2 and BC_2F_2 crop-weed hybrids were not significantly different from wild QC2974 for vegetative productivity (Contrast, $P=0.2$). No significant difference for reproductive productivity was detected between different plant types (ANOVA, $P=0.65$) (Fig. 7). Westar and BnGT1 produced similar number of seeds per plot to wild and transgenic *B. rapa* plants; however, both had significantly higher vegetative dry weight than wild and transgenic *B. rapa* plants.

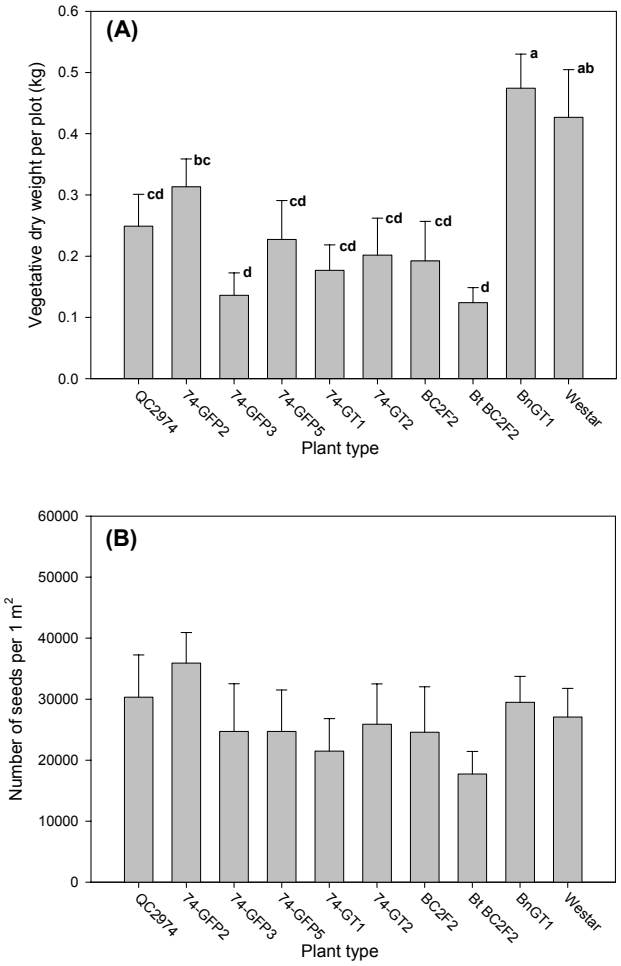


Figure 7 Vegetative and reproductive productivity of transgenic *B. rapa* events under field conditions. Wild QC2974, 74-GT and GFP events, BC_2F_2, Bt BC_2F_2 hybrids, and *Brassica napus* (BnGT1 and non-transgenic *B. napus*) were planted and grown under field conditions with few herbivorous insect pressure. Panel (A) represents vegetative productivity, and panel (B) represents reproductive productivity. Different letters in panel (A) represent significant differences between different plant types (ANOVA, $P<0.05$). No significant difference for vegetative fitness was detected between different plant types (ANOVA, $P = 0.65$). Error bars represent $\pm$ standard error of the mean.

Discussion

Transformation of weedy B. rapa accessions

B. rapa, which commonly grows in or near *B. napus* cultivated areas, is known as one of the most recalcitrant members of the *Brassica* genus to regenerate shoots *in vitro* (Murata and Orton 1987; Narashmhulu and Chopra 1988). Several *B. rapa* crop types, such as *oleifera*, *chinensis*, and *pekinensis*, have been transformed via *Agrobacterium*-mediated methods (Kuvshinov et al. 1999; Wahlroos et al. 2003; Qing et al. 2000; Zhang et al. 1998). This paper describes the first transgenic weedy accessions of *B. rapa* to have been produced and characterized. Relatively low transformation efficiency of *B. rapa* was reported (i.e. ca. 0.4% efficiency—nearly one-tenth the rate of our typical transformation efficiency with *B. napus*). Transformation efficiency may have been influenced by several factors, including genotype, explant type, donor plant age, and the *Agrobacterium* culture parameters (Poulsen 1996). Kuvshinov et al. (1999) showed that the shoot recovery efficiency of *B. rapa* spp. *oleifera* was highly dependent on the tissue used as explants. Another possibility is that wild weedy *B. rapa* may be recalcitrant to transformation *in vitro* because of its weedy genetic background and the tissue culture and transformation conditions have never been optimized for weedy genotypes.

Characterization of transgenic B. rapa events

Relatively high GFP expression T_2 plants were selected by visual assay for characterizations. These selected T_2 plants were assumed to be homozygous plants for the GFP gene, since homozygous and hemizygous could be differentiated by visualization of different GFP intensity in *Nicotiana tabacum* seedlings (Molinier et al.

2000) and *B. napus* (Halfhill et al. 2003a). The 74-GFP5 event that was all GFP positive in the T_1 generation may have multiple transgene integrations that occurred on both chromatids of the same chromosome. Inheritance of GFP and Bt transgenes in T_2 generations of transgenic *B. rapa* events were confirmed by measurement of GFP expression and Bt ELISA analysis. Many possible factors can account for varying intensity of GFP fluorescence, including positional effect of inserted transgene, leaf age, or unknown physiological variability (Molinier et al. 2000; Halfhill et al. 2001; Halfhill et al. 2003b). Fluorescence intensity at 508 nm varied among the independent transformation *B. napus* events (Halfhill et al. 2003b).

Zhu *et al*. (2004) reported that Bt transgene expression was stable and persistent in F_1 and subsequent backcrossed *B. rapa* populations. Different Bt concentrations of GT *B. rapa* events is likely the result of position effects and number of transgenes inserted. Although previous studies in transgenic GT *B. napus* events strongly associated GFP fluorescence intensity with Bt concentration at maturity (Halfhill et al. 2003b), in this study no correlation was detected between the GFP fluorescence and Bt concentration in transgenic GT *B. rapa* events. This discrepancy with previously described transgenic events could be a matter of sampling at different plant age, as all our transgenic plants were analyzed 24 days after planting. No correlation between copy number of transgenes and GFP fluorescence intensity was observed which contrasts with Stewart et al. (1996) for Bt-transgenic *B. napus*. Another study by Hobbs et al. (1993) has reported that copy number of GUS transgene in tobacco was associated with transgene expression either positively or negatively.

Toxicity of Bt transgene to diamondback moth (DBM)

Insecticidal genes such as Bt *cry1Ac* have been used to genetically engineer many agricultural crops (Schuler et al. 1998). Several Bt crystal protein endotoxins have been proven effective in reducing insect damage in crop plants (Hofte and Whiteley 1989). Bt susceptible diamondback moth strain was used in this study, however, several Bt *cry1Ac* resistant diamondback moth strains were developed (Roush 1994; Metz et al. 1995; Tabashnik et al. 1993). Bt transgenic OSR can be effective for management of diamondback moth (Ramachandran et al. 1998a,b,c). However expression of Bt transgene in weed populations may increase the difficulty of weed control under insect selection pressure. Transgene escape from crop species into wild weedy populations has been observed, and the fitness or productivity of crop-weed hybrid populations caused by transgene escape has been studied (Hauser et al. 1998a; Gueritaine et al. 2002; Warwick et al. 2003; Mason et al. 2003; Halfhill et al. 2005). In this study, expression of Bt gene in Bt transgenic weedy plants and crop-weed hybrids was effective in limiting damage caused by DBM. We concluded that the protection of the Bt transgene stabilized productivity of transgenic *B. rapa* plants or crop-weed hybrid populations under herbivore pressure. However, it was apparent that as in Halfhill et al. (2005), interspecific hybridization was a greater factor affecting productivity than transgenic status. In that prior study, it was noted that one missing plant type that would be useful for comparisons was a directly transformed *B. rapa* that is the subject of this study.

Transgenic B. rapa productivity under greenhouse and field conditions

Seed production from Bt transgenic OSR in plots infested with DBM was 15 times higher than the non-Bt oilseed rape (Ramachandran et al. 1998c). However, in our greenhouse study, Bt transgenic plants produced about twice as many seeds as the non-transgenic plants. This maybe explained by the insufficient number of applied DBM or insufficient frequency of DBM application to cause large differences. In the field trial, few herbivorous insects were observed during the experiment. Crop-weed hybrids, with or without the insecticidal transgene, had similar productivity as both directly transformed *B. rapa* and wild *B. rapa* in the absence of herbivorous insect field conditions. However, crop-weed hybrids containing the Bt transgene have had significantly less reproductive productivity than their non-transgenic parents in the absence of herbivore pressure (Vacher et al. 2004; Halfhill et al. 2005). The material generated in this study will be valuable in future field experiments for testing the influence of insect pressure on productivity in transgenic *B. rapa* and crop-weed hybrids.

Directly transformed weedy lines will act as ultimate controls to assess the genetic load of crop genes moving into weedy populations. In the case of the directly transformed weed, the transgene of interest lies in the midst of other weedy genes, which contrasts with the case of introgressed transgenic weeds. The latter transgenes are surrounded by crop genes (Halfhill et al. 2003a; Stewart et al. 2003). In the current study, the directly transformed weeds had equivalent productivity to the non-transgenic weeds. We observed little variation among transgenic *B. rapa* events for production under mild insect pressure, indicating that any might be suitable for comparative field experiments.

We will perform advanced field studies with these plants to continue to better define the risk that introgression plays in transgenic agriculture.

Chapter Three

Expression of green fluorescent protein (GFP) in pollen of oilseed rape (*Brassica napus* L.) and direct measurement of pollen dispersal*

* This chapter has been submitted to Theoretical and Applied Genetics with authors Hong S. Moon, Matthew D. Halfhill, Laura C. Hudson, and C. Neal Stewart, Jr.

Abstract

Transgene movement via pollen is an important parameter for understanding and evaluating possible out-crossing capacities of transgenic crop varieties. Here, we describe the movement of canola (*Brassica napus* L. cv. Westar) pollen expressing a genetically encoded fluorescent tag. Transgenic canola plants were produced using an *Agrobacterium*-mediated transformation method with the pBINDC1 construct containing a green fluorescent protein (GFP) variant, *mGFP5-ER*, under the control of the pollen-specific LAT59 promoter. Transgenic pollen was differentiated from non-transgenic pollen *in vivo* by a unique spectral signature and was shown to be an effective tool to monitor pollen movement in proof-of-concept studies in the greenhouse and field. GFP-tagged pollen also served as a practical marker to determine the zygosity of plants. In a

greenhouse study, more pollen was captured at closer distances from the source plant plot with consistent wind generated by fans. Under field conditions, GFP transgenic pollen grains were detected up to a distance of 15 meters. No significant differences were detected under field conditions for pollen frequency among distances 0, 5, 10, and 15 m from the source plant plot, and no consistent wind effects were detected on the number of pollen grains on pollen traps. No significant differences between transgenic pollen and non-transgenic pollen were detected for pollen dispersal under field conditions.

Introduction

Pollen flow is a prominent mode for transgene movement in the environment, and it is desirable to track transgene movement under field conditions to assess potential ecological risks such as the interspecific hybridization with weedy relatives (e.g. Warwick et al. 2003). The pattern of pollen movement from a transgenic crop variety is a direct measure of out-crossing potential to conspecific crops and wild relatives. To date, most gene flow studies have been performed by progeny analysis using conventional molecular techniques. Although these studies reflect actual hybridization events, the capacity of the pollen to move within the environment has been measured indirectly, with little information on pollination vector (i.e., wind or insect). We proposed that green fluorescent protein (GFP) expressed in pollen grains may be used as a marker to directly measure pollen movement within the environment (Hudson et al. 2001), and here we test this proposal in an agronomic crop with the propensity for intra-and interspecific hybridization: *Brassica napus*.

Oilseed rape (*Brassica napus* L. AACC 2n=38) transgene flow research under field conditions has often been performed using an herbicide tolerance trait as a detection method (e.g., Damgaard and Kjellssion 2005) that requires seed collection and progeny analysis using a destructive method. Green fluorescent protein (GFP) can replace conventional molecular techniques and herbicide resistant markers as a real-time *in vivo* marker for the presence and expression of transgenes (Stewart 1996, 2001, 2005). Using GFP expressed in vegetative tissues under the control of the CaMV 35S promoter, transgene flow has been assessed in transgenic oilseed rape using progeny analysis (Halfhill et al. 2001); however, GFP was not expressed in the pollen. A system including pollen tagged with GFP could be used in monitoring transgene movement to better understand pollen distribution biology. The tomato LAT59 promoter (Twell et al. 1991), which is preferentially expressed in the anthers and pollen of tomato, was effectively used to express GFP in tobacco pollen (Hudson et al. 2001). Using pollen traps to measure pollen movement from transgenic varieties has been considered an inappropriate technique because it is almost impossible to distinguish from one variety of pollen from another (Wang et al. 2004). GFP-tagged pollen may allow researchers to distinguish between the pollen of transgenic and non-transgenic plants within the same species in relatively complex environmental mixtures.

Oilseed rape is an appropriate crop for the study of transgene escape or movement, since it has many wild relatives such as birdseed rape (*Brassica rapa* L.) which occurs as weed populations in or near canola cultivation areas and has an overlapping flowering period (Simard et al. 2002; Halfhill et al. 2002; Chevre et al. 2003). Oilseed rape is a partially self-fertilizing species, at rates ranging from 53-88%

depending on cultivar and environmental conditions (Becker et al. 1992). Approximately half of oilseed rape pollen grains, which are heavy and sticky, have been found to fall onto plant surfaces and the ground within 3 m from the source plant (Lavigne et al. 1998). A small percentage of oilseed rape pollen becomes airborne and can move via the wind (Lavigne et al. 1998). In addition, insect pollinators, especially bees, can transmit oilseed rape pollen for long distances. Pollen dispersal of oilseed rape up to 3 km has been reported (Rieger et al. 2002).

In this study, we describe the generation of transgenic oilseed rape lines that express GFP in pollen grains, and demonstrate that GFP pollen dispersal patterns can be detected under greenhouse and field conditions. GFP fluorescence in pollen grains also allowed the determination of the zygosity of transgenic oilseed rape plants by the segregation of GFP-tagged pollen and inferring the parent's zygosity status. The LAT59 promoter coupled to GFP may serve as a practical marker to track pollen movement directly for risk assessment research.

Materials and Methods

Plant transformation

Plant transformation and regeneration methods were based on an existing protocol (Stewart et al. 1996). Plant transformation was carried out with oilseed rape (*B. napus* L. cv. Westar). The pBINDC1 construct (described in Hudson et al. 2001) that contains the mGFP5-ER variant under the control of the LAT59 pollen specific promoter was used for *Agrobacterium*-mediated transformation. All cultures were maintained at 24±2ºC under a

16/8hr (light/dark) photoperiod. The recovered number of explants, shoots, rooted shoots, and fertile plants were recorded. Transformed oilseed rape events with the pBINDC1 plasmid were designated as LH *B. napus*.

Zygosity determination

Twenty-six T_1 transgenic seeds from the transformed oilseed rape event 1 (LH1) were planted and grown in the greenhouse. Five flowers were collected separately from each individual T_1 LH1 plant to assay pollen. Collected flowers were tapped by hand on clean microscope slides (Fisher Scientific, Pittsburgh, PA, USA) to collect the pollen. Collected pollen from each individual plant was observed using an epifluorescent (FITC filtered) microscope (Olympus BX51 model) with blue light excitation at 200x magnification to score pollen for GFP and infer zygosity status of the parent. The pollen population was inferred to come from homozygous, hemizygous, or non-transgenic isogenic lines for the GFP transgene based upon Mendelian expectations and observations.

Greenhouse experiment

A greenhouse experiment was conducted in the Racheff research greenhouse at the University of Tennessee at Knoxville, USA. Homozygous LH1 and Westar were planted in 4 L pots and placed alternately. In total there were 15 transgenic and 15 non-transgenic plants in the experiment. The LH1 and Westar plants were set up in the greenhouse with a fan to generate air currents (Aloha 30" Pedestal fan). Pollen dispersion was measured when each individual plant had more than 30 open flowers. Pollen traps were

constructed by covering microscope slides with petroleum jelly and attaching them to wooden stakes with twist ties. The slides were attached to wooden stakes at 1 m from the greenhouse floor. Pollen traps were placed at 2, 4, 6, 8, 10, and 12 m from the source plants. Wind speed was measured at each distance using a portable wind meter using the 'Avg10' function, which allowed the measurement of average wind speed for 10 seconds (Skymate, Speedtech Instruments, Great Falls, VA, USA). New pollen traps were placed at 9:00 am in the morning and collected at 5:00 pm within the same day. Pollen collection was conducted for 3 consecutive days. Collected pollen traps were assessed under an epifluorescent microscope with blue light. The number of LH1 and Westar pollen grains in each pollen trap was recorded.

Field experiment

A field experiment was performed at the Knoxville Experiment Station, Knoxville, TN, USA (35°58'N, 83°55'W) in the spring of 2005. The array of plots was based on Saeglitz et al. (2000) (Fig. 1). Each center quadrant was 3 m^2 and contained 150 plants. Methods used in the field experiment for the construction of pollen traps were the same as described in the greenhouse study. Pollen traps were placed in different directions (N, S, E, W, NW, SW, NE, SE) at distances of 0, 5, 10, 15 m from the center plant source plot. Fresh pollen traps were placed at 8:30 am and collected at 5:30 pm within the same day. Pollen traps were collected for 4 consecutive days. Wind direction and speed were acquired from the wind information recorder located at the Knoxville Experiment Station. Collected pollen traps were screened under an epifluorescent microscope with blue light

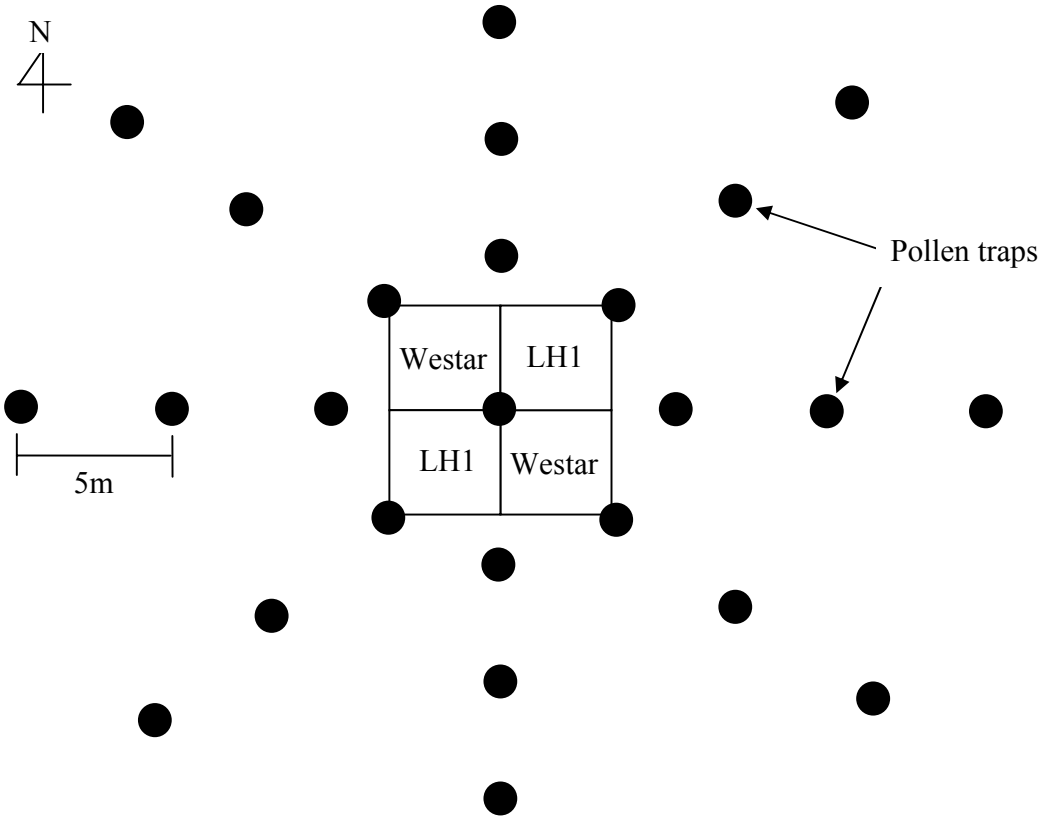


Figure 1 Field design consisted of a central plot split into 4 quadrants. Two quadrants contained LH1 and the remaining two contained Westar. Pollen traps were placed in different directions (N, S, E, W, NW, SW, NE, SE) at distances of 0, 5, 10, 15 m from the center of the source plant plot.

at 200x magnification. The numbers of LH1 and Westar pollen grains were recorded in each pollen trap.

Results

Plant transformation

Two LH *B. napus* events were regenerated from separate callus sectors using the *Agrobacterium*-mediated transformation method. Out of 1,024 explants, 7 shoots were recovered. From these recovered shoots, 3 formed roots. Two rooted T_0 plants produced T_1 seeds and the T_1 generation plants were phenotypically identical to Westar by visual inspection.

Zygosity determination

The zygosity of plants was determined based on the GFP pollen to non-GFP pollen ratio under epifluorescent microscopy using a FITC filter set. T_1 generation seeds from self-pollinations were germinated, and these plants were categorized as homozygous, hemizygous, and isogenic lines for the transgene according to the frequency of GFP expression in the pollen (Fig. 2). Both transgenic events apparently harbored transgenes in single loci based upon pollen segregation. Homozygous T1 plants had all transgenic pollen and hemizygous plants had 50% transgenic pollen as was observed in single-locus tobacco transgenic for the same construct (Hudson et al. 2001).

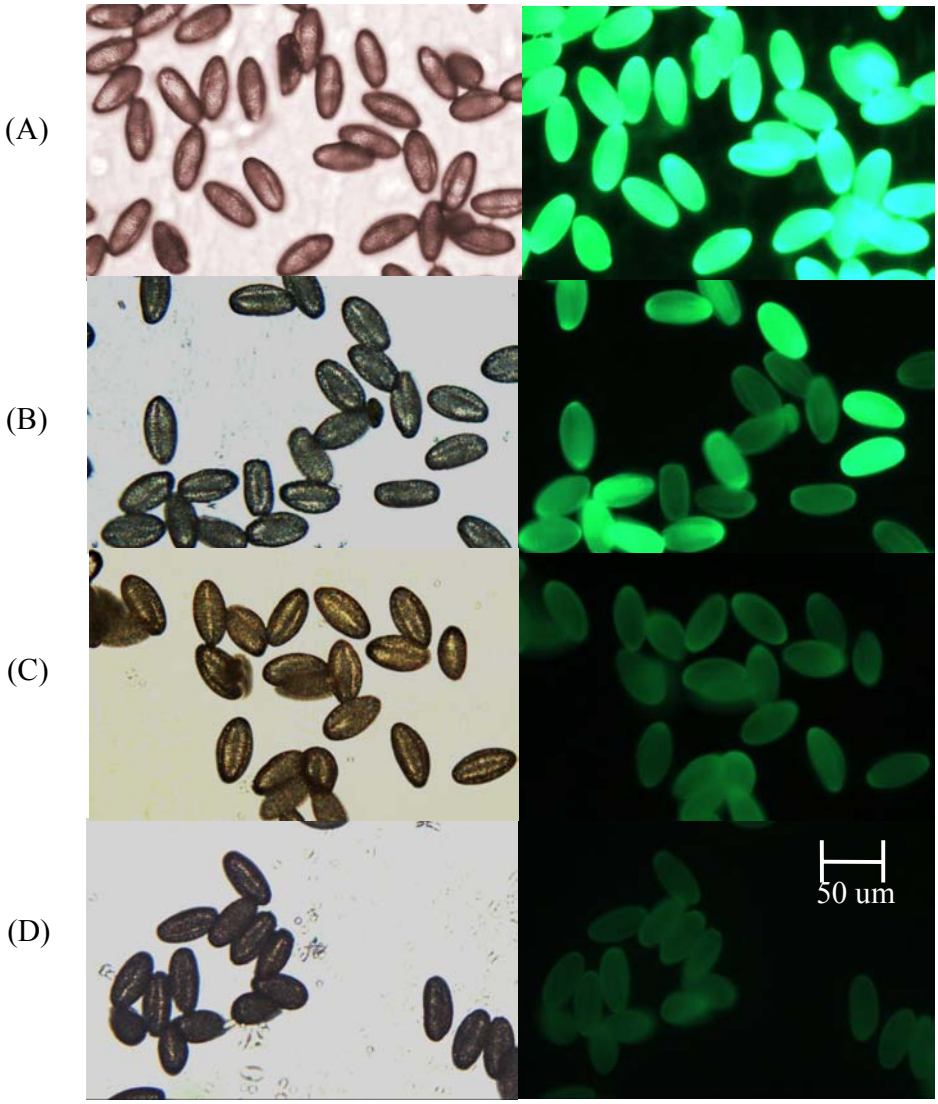


Figure 2 Determination of zygosity based on green fluorescent pollen. Pollen of T_1 LH1 and Westar. (A), (B), and (C) represent pollen of homozygous, hemizygous, and isogenic LH1 respectively. (D) represents Westar pollen. These pictures were taken under white light (left column) and blue light (right column) with exposure times of 16.7 ms and 1.54 s respectively with 200x magnification.

Greenhouse experiment

Pollen grains detected in the pollen traps were categorized into GFP-tagged transgenic and non-transgenic pollen. Ninety-two GFP-tagged pollen grains were detected at 2 m from the source plants on each of 3 consecutive days. GFP-tagged pollen grains were distinguished from non-transgenic pollen grains using blue light under the epifluorescence microscope. Pollen traps placed at closer distances to the center source plants trapped more pollen from both LH1 and Westar (Table 1). LH1 pollen traveled up to 10 m from the source plants; however wind from the fan was not detected at the 10 m distance. Westar pollen was detected at a distance of 8 m from the source plot on the second day.

Field experiment

Over 4 consecutive days, 417 LH1 pollen grains and 583 non-transgenic Westar pollen grains were detected. Collected pollen traps were screened and LH1 pollen grains were identified by visual assay of GFP fluorescence under an epifluorescent microscope with blue light. GFP-tagged pollen grains were distinguished from non-transgenic pollen grains by GFP fluorescence. No significant difference was detected for the number of pollen grains per trap on average between LH1 and Westar (ANOVA, P=0.34). The number of pollen grains collected on traps from LH1 and Westar in each direction on each day varied; however, no significant difference was observed between the number of pollen grains collected for each distance (ANOVA, P=0.26) (Fig. 3). Significant differences were shown for the number of pollen grains per trap among different directions (ANOVA, P<0.05) (Fig. 4). No consistent effect of wind direction or wind

Table 1 Number of pollen grains collected from LH1 and Westar under greenhouse conditions. Distance refers to meters from the source plants to pollen traps. Experiment was performed for 3 consecutive days. Detected pollen was screened and counted under epifluorescent microscope with blue light.

Distance (m)	Wind speed (m/s)	1st day		2nd day		3rd day	
		Westar pollen	LH1 pollen	Westar pollen	LH1 pollen	Westar pollen	LH1 pollen
2	0.76	99	58	85	4	0	30
4	0.58	78	0	1	0	0	0
6	0.36	9	0	0	0	0	0
8	0.18	0	6	12	0	0	1
10	0	0	0	0	1	0	0
12	0	0	0	0	0	0	0

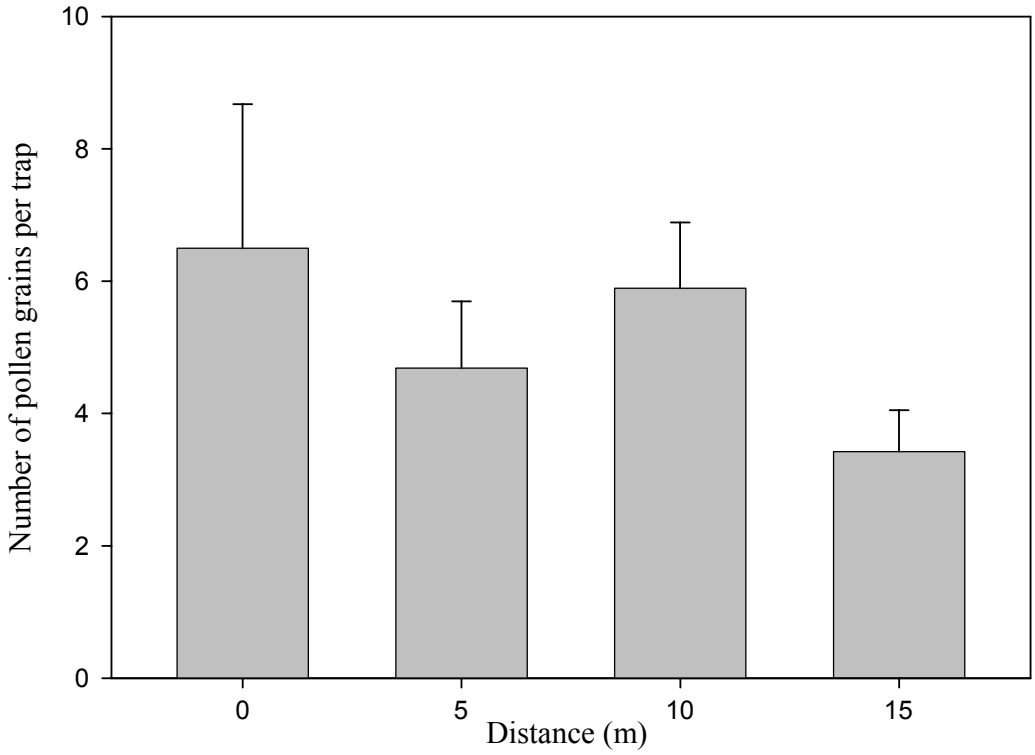


Figure 3 Average number of pollen grains detected per day at each distance under field conditions. Distance indicates the distance from the center of the source plant plot. Standard errors of the mean are shown. No significant difference was detected among distances (ANOVA, P=0.26).

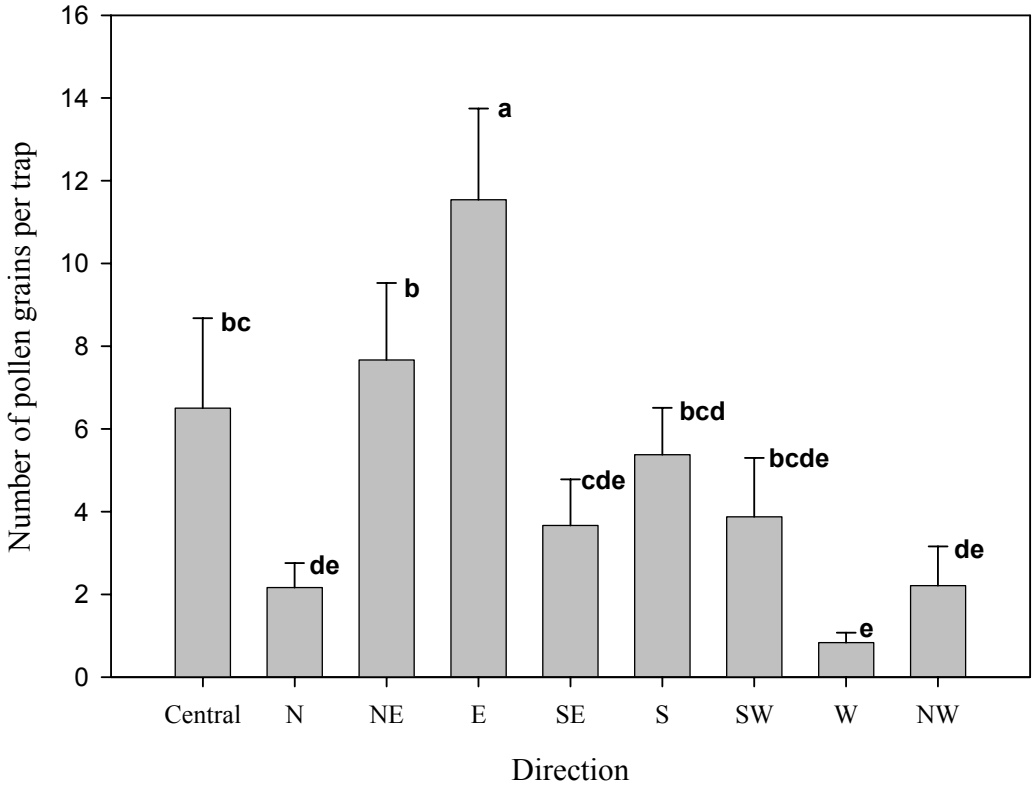


Figure 4 Average number of pollen grains collected in different directions under field conditions. Central indicates the average number of pollen grains collected in 2 pollen traps at the center of the plot. The remaining directions represent the number of pollen grains collected on average of 3 pollen traps at 5, 10, and 15 m. Standard errors of the mean are shown. Different letters represent significant differences for the number of pollen grains between directions (ANOVA, Fisher's LSD, $P<0.05$).

speed for number of pollen was detected (Fig. 5). On day 1, most wind blew toward the west direction and speed ranged from 0.29 to 5.16 m/s, however, no pollen grains were detected on pollen traps placed west from the center plot. On day 3, most wind blew between northeast and southeast, and pollen traps placed east from the source plot caught more than 10 pollen grains on average per trap.

Discussion

Transgenic plants have been used as an effective tool to quantify gene flow (Messeguer 2003). Among marker genes used in previous transgenic research, GFP is among the most suitable *in vivo* markers to track transgene movement via pollen for the purpose of risk assessment purpose and to study reproductive biology. GFP transgene is a neutral reporter gene and non-toxic to plants, making it especially useful for studying the development of functional pollen (Harper et al. 1999; Stewart 2001; Ottenschlager et al. 1999; Hudson and Stewart 2004). In tobacco (*Nicotiana tabacum*), when GFP is expressed under the control of the pollen- specific LAT59 promoter, pollen fitness and tube germination frequencies were not different from pollen of non-transgenic plants (Hudson and Stewart 2004). The LAT59 promoter coupled with the GUS gene in plant showed high GUS expression in pollen but low level of GUS expression in roots and seeds (Twell et al. 1990). GFP-tagged pollen provides an efficient method to differentiate between transgenic and non-transgenic pollen from the same species (Hudson et al. 2001).

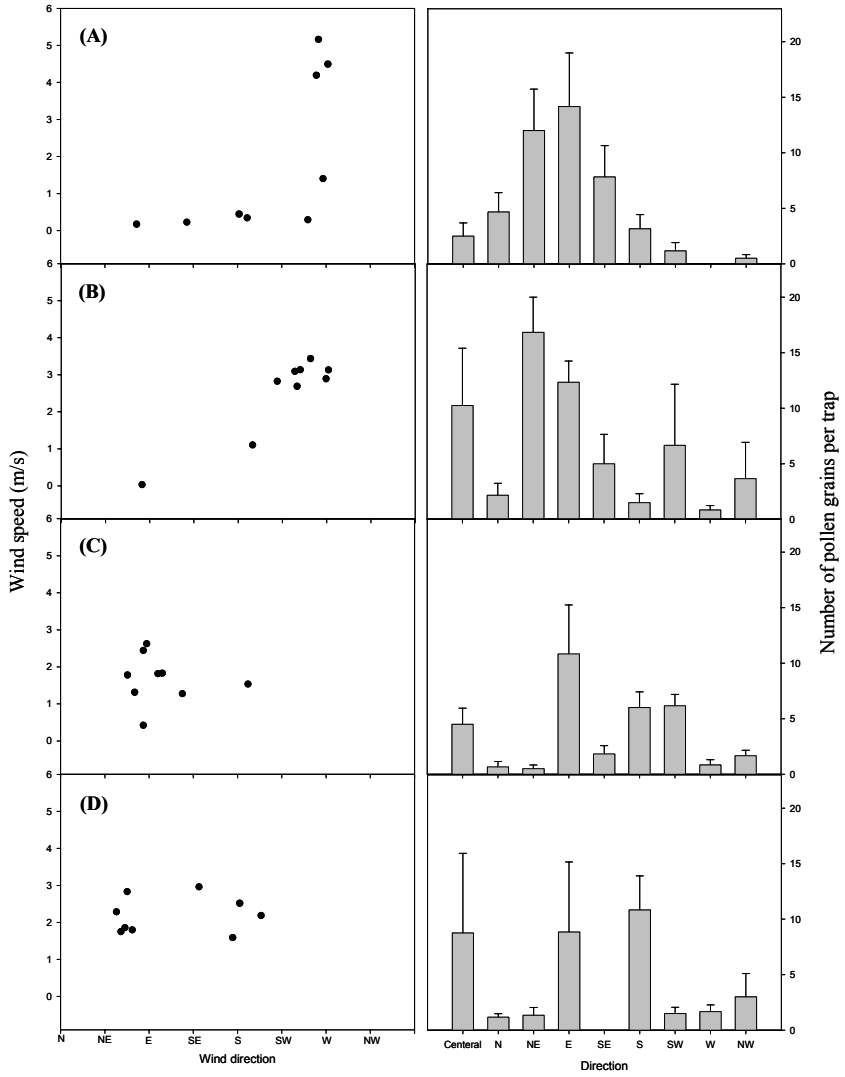


Figure 5 Number of pollen grains collected under field conditions and wind information. Pollen grains were collected in each direction on each day with wind direction and speed measured every hour from 9:00 am to 5:00 pm. (a), (b), (c), and (d) represent wind direction and speed measured at every hour and average number of pollen grains collected from pollen traps for each direction. (a), (b), (c), and (d) represent day 1, day 2, day 3, and day 4 respectively. Standard errors of the mean are shown.

Determining the transgene zygosity status of individual plants is important in plant breeding, and GFP-expressing pollen may be another tool to enable this process. Zygosity in transgenic plants can be visualized based on difference in the intensity of GFP fluorescence when driven by the constitutive 35S CaMV promoter (Molinier et al. 2000; Halfhill et al. 2003b). Also, zygosity in transgenic plants can be analyzed by real-time PCR (German et al. 2003; Bubner and Baldwin 2004). The method using the LAT59::GFP construct was shown here to be an efficient method for determination of zygosity in transgenic plants. Our GFP-tagged pollen method requires relatively little time to prepare the samples and is a rapid, reagent-free alternative to other methods such as real-time PCR.

Agrobacterium-mediated transformation has been the most common method of gene transfer in oilseed rape (DeBlock et al. 1989). Relatively low transformation efficiency (0.16%) was shown from oilseed rape transformation with the pBINDC1 construct, because transformation efficiency may have been influenced by several factors such as cultivar, donor plant age, explant type, experimental conditions, and the *Agrobacterium* culture parameters (Poulsen 1996).

Oilseed rape normally produces an abundant amount of pollen for approximately 4-5 weeks (Damgaard and Kjellsson 2005). The majority of the pollen is dispersed over a short distance (Lavigne et al. 1998). In commercial oilseed rape fields, cross-pollination occurred at higher frequencies at short distances from the source field (Rieger et al. 2002). The greenhouse pollen dispersal study is important because several factors that can have an effect on pollen movement such as wind speed and direction could be controlled and kept consistent during the experimental process. In the greenhouse study,

more pollen was detected at shorter distances from the pollen source plants. Scheffler et al. (1993) estimated the frequency of oilseed rape pollen dispersal to be approximately 4 times higher at a distance of 1 m than at 3 m from the transgenic pollen source when surrounded by non-transgenic plants acting as recipients at varying distances. However, in our study, no significant differences in the number of pollen grains were found in the traps at different distances (0, 5, 10, and 15 m) from the source plant plot under field conditions. The estimation of pollen frequency by Scheffler et al. (1993) may be explained by the close proximity of non-trasngenic plants to the pollen source plot creating a buffer zone and then preventing the spread of transgenic pollen to greater distances.

Wind direction and number of pollen grains were not correlated. Four days might not be a sufficient period to prove a correlation between wind directions and pollen flow. Wind direction and speed were recorded every hour from 9:00 am to 5:00 pm, so wind direction and speed were not known between the hourly measurements. In future studies, continuous measuring of wind direction and speed may be required to detect a correlation between number of pollen grains and either wind direction or wind speed. Also, larger experimental plots generating larger pollen clouds would provide more realistic dispersal patterns and may allow for the upper limit of pollen flight to be detected experimentally.

Other constitutive promoters, such as the maize *Adh1* promoter, which have shown activity in root, shoot meristems, and pollen (Kyozuka et al. 1991) could be potential candidates for future risk assessment studies in monocots. There are no promoters that regulate strong constitutive expression to that degree in dicots. In this

study, GFP under the control of the LAT59 promoter allowed pollen to be distinguishable from the same species based the GFP expression in the pollen. Experimental improvements are also possible to enhance the ability to detect pollen movement via fluorescence. For example, GFP might not be the optimal fluorescent protein to tag pollen because of autofluorescence in blue light (Stewart 2005). Other fluorescent proteins such as a red fluorescent protein (RFP) might be good markers for expression in plants (Eckert et al. 2005; Stewart 2005) including pollen grains having different colors from non-transgenic pollen of oilseed rape. Also, new technology such as laser-induced fluorescence spectroscopy (Stewart et al. 2005) that allows visualization of GFP in ambient light may be a more efficient tool to detect GFP-tagged pollen movement. GFP-tagged pollen may be an efficient tool to directly track transgene movement via pollen in real-time for the purpose of risk assessment in ecological studies.

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