This is to certify that the

dissertation entitled

Risk Assessment of Transgenic Plants: Evaluation of Border Rows as a Containment Strategy for Transgenic Pollen and a Comparison of Pollen Dispersal Patterns for Native and Transgenes

presented by

Stan C. Hokanson

has been accepted towards fulfillment
of the requirements for

PhD degree in Horticulture
Plant Breeding and
Genetics Program
Ecology and Evolutionary
Biology Program

J F Hancock / Rebecca Grumet
Major professor

Date 12/21/95

RISK ASSESSMENT OF TRANSGENIC PLANTS: EVALUATION OF BORDER ROWS AS A CONTAINMENT STRATEGY FOR TRANSGENIC POLLEN AND A COMPARISON OF POLLEN DISPERSAL PATTERNS FOR NATIVE AND TRANSGENES

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Horticulture
Plant Breeding and Genetics Program
Ecology and Evolutionary Biology Program

1995

ABSTRACT

RISK ASSESSMENT OF TRANSGENIC PLANTS: EVALUATION OF BORDER ROWS AS A CONTAINMENT STRATEGY FOR TRANSGENIC POLLEN AND A COMPARISON OF POLLEN DISPERSAL PATTERNS FOR NATIVE AND TRANSGENES

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Despite full commercial approval of twelve transgenic crops in the U.S. (circa 1995), concern is still being expressed regarding the potential risks associated with the agronomic-scale production of transgenic crops. One commonly mentioned concern involves the pollen-mediated escape of engineered genes into populations of crop wild relatives. In this study two questions relevant to this issue were investigated: 1) Can plantings of border rows effectively limit pollen mediated gene movement, and 2) Do the pollen-mediated dispersal patterns of transgenes differ from those of native genes? The ratio of recessive trap plants to wild type donor plants was varied to test the efficacy of border rows as a means to limit the spread of transgenic pollen to discontiguous satellite plots. Gene movement within the border plots assumed a leptokurtic distribution. Increasing the number of donor plants increased levels of gene flow both within the border and to the discontiguous satellite plots. As the trap/donor ratio increased, there was a significant decrease in long distance gene movement to the satellites, although the observed year to year and site to site variability could limit the effectiveness of this strategy. Furthermore, extremely large numbers of border plants would be required to minimize pollen movement on a

commercial scale. Dispersal patterns of transgenes and native genes were evaluated by comparing levels of pollen-mediated gene movement from melon plants (*Cucumis melo*) expressing dominant morphological and transgenic marker genes into a surrounding border of recessive non-transgenic melon plants. Long distance dispersal patterns for the two genes were identical and dispersal patterns into the plot borders were nearly identical. Several of the apparent discrepancies were explained by transgene inactivation, a phenomenon which has implications for any study measuring gene movement with transgenic plants. Results from this study validate the assumption that native and transgenes have the same dispersal patterns. Thus, application of non-transgenic results to transgene escape and dispersal issues should be appropriate. However, the assessment of establishment and spread will depend on both pollen movement and the fitness value of the particular transgene crop combination.

ACKNOWLEDGEMENTS

I was born in the age of rockets and grew up on a dirt road between two small towns. In large part this seemingly insignificant juxtaposition of two worlds has led me here to the point of completing this project. Perhaps what I mean to say is, finishing this project represents the culmination of a long journey. However, the task at hand is not to explain the philosophical basis of my scientific life (which I probably could not accomplish anyway). Rather, my intent here is to attempt to acknowledge the people who have made this chapter of my life possible. To name everyone would be nearly impossible and yet at some risk I will name a few. The mere mention of these names in no measure expresses my gratitude. In the same vein, my failure to mention here all who played a role in this chapter of my life does not diminish their role. If you sweated, fretted, laughed or cried with me along the way, the depth of that emotional bonding is the true measure of my gratitude

Early on several people strongly encouraged me to go back to school. My mother has always been my staunchest advocate. Her unwavering faith in me helped start this ball rolling. My good friends Dick and Deb Rieth and Ken Kirton have never failed to love and encourage me as I expect they never will.

Once in graduate school the friendships came fast and furiously with all the help and encouragement one could ever hope for. My friends Pete and Mollie Callow offered me a place to stay and warm meals when I was but a name on paper. That early act of kindness has blossomed into a fast friendship. From the beginning Steve Krebs took me under his professional wing, providing both practical advice and very enjoyable eclectic discussion. He and his wife Roberta have become a constant source of friendship and encouragement. Roger and Lori May have shared the trials and tribulations of this graduate student life cheerfully and generously. The comradery, help and encouragement they provided was boundless. At times, Sue Hammar all but carried me in the lab. A similar role was played by Dan Prince and John Holmes in the field and Camille Ciesliga in the greenhouse.

I thank my committee members both past and present; Steve Tonsor, Steve Malcolm, Bernie Zandstra, and Andy Jarosz for the thoughtful reviews of my manuscripts and dissertation and their non-threatening bearing which made this process more enjoyable and rewarding.

My advisors Rebecca Grumet and Jim Hancock demonstrated an extraordinary concern for my progress as a graduate student. Rebecca's precision thinking and writing skills served me well at all times. However, her desire to excel in all aspects of the student/advisor process was an inspiration for me.

Jim Hancock took me into his lab when all I could do was hoe grapes and speak vaguely of my aspirations. He has delivered me to the threshold of those aspirations. Over the years Jim has served in all the typical advisor roles and some not so typical. He has been a dependable training partner and a wonderful friend. I

have seen him take the risk of becoming involved in the personal/emotional aspects of people's lives, delivering a human element to this process we call higher education. Jim's natural tendency to see the good in the people and situations around him gives him the ability to draw out the best in those around him without intimidating or pressuring. Jim also has an uncanny ability to clearly see the fulcrum on which issues reside. This ability has simplified many issues for me over the years. I want to thank Jim for providing a non-threatening environment in which I could learn how I wanted to do science and find my new path in this world!

Last I thank my wife Karen and daughter Kate who have given me a whole life. From them I have learned lessons I never expected to learn in this institution of higher learning. Karen has become my best friend in this world. Her ability to carry so many tasks forward with such quiet efficiency and grace has won my undying admiration. Her spirit has won my undying love.

Guidance Committee:

The journal paper format was chosen for this thesis in accordance with departmental and university regulation. The thesis is divided into three chapters and two appendices. Chapter 1 is in press in HortScience. Chapter 2 has been submitted for review to Ecological Applications. Chapter 3 will be submitted to Theoretical and Applied Genetics. Appendix 1 and 2 are intended as a guide for future herbicide selection experiments planned as an extension of the work presented herein.

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INTRODUCTION

The first breakthroughs in genetic engineering technology occurred in the early 1970's. Included among the many breakthroughs of this era were the ability to clone DNA in a bacterial host or vector (1973), the discovery of restriction enzymes capable of cutting DNA at specific sites (1975) and the ability to determine the exact base sequence of a DNA fragment (1975). In 1983 researchers at the university of Ghent and at the Monsanto Co. had independently uncoupled the crown gall causing genes from the Ti plasmid of *Agrobacterium tumefaciens* (Zambryski et al., 1983; Fraley et al., 1983). By replacing the tumor inducing region with a DNA sequence of interest they had created a bacterium capable of transfering foreign DNA into the plant genome and thus was born the era of plant genetic engineering. Since that time progress in the development and testing of transgenic plants has been dramatic. Today approximately 60 plant species have been or are currently in the process of being genetically engineered (Raybould and Gray, 1993; Rissler and Mellon, 1993; Rissler and Mellon, 1994; Rogers and Parks, 1995). Moreover, twelve transgenic crop/gene combinations have now been given full commercial approval (Rissler and Mellon, 1995). It has been suggested that genetic engineering will become the fourth wave in modern food production following selective breeding, modern hybrids and the use of petrochemicals (Gasser and Fraley, 1989; Fraley, 1992). However, commensurate with

fast development of the technology, and the excitement generated by the potential benefits to be provided by it, concern has been voiced regarding the potential risks associated with the production and consumption of genetically engineered crops on a commercial scale.

These concerns fall into three general categories; 1) the potential risk posed by transgenic crops to human health, 2) the potential risk posed to the domesticated plants and animals on which we depend, and 3) the potential risk that is posed to the natural environment by genetically engineered crops. There are a number of potentially negative interactions which could occur between genetically engineered crops and natural ecosystems. The research described within this dissertation addresses the general area of the potential risk of pollen-mediated escape of transgenes from crop plantings into natural populations of crop wild relatives.

Gene flow has long been recognized as an elemental evolutionary force. Migration of a few individuals between small populations can offset the effects of random drift and selective pressures (Wright, 1951; Antonovics, 1968). Much effort has been directed toward understanding the mechanisms and implications of gene flow in both a theoretical and empirical sense. Along these lines, one of the most prominently mentioned risks associated with the commercialization of transgenic crops has been the potential for pollen-mediated escape of engineered genes (Colwell et al., 1985; Ellstrand, 1988; Ellstrand and Hoffman, 1990; Dale, 1992; Raybould and Gray, 1994).

If established in natural populations, transgenes could effect population dynamics within the species complex itself or at the community level. At the species

level a transgene conferring a high fitness value to it's carrier could cause a major shift in allele frequencies within the population raising the possibility of the loss of rare alleles not associated with the transgene (Regal, 1994). Even in the absence of a selective advantage, "swamping" of small natural populations with domesticated genes from large agronomic plantings could lead to the extinction of many "wild" genes (Ellstrand, 1992), or species (Small, 1984). Species loss could include crop wild relatives in the centers of crop diversity (Rissler and Mellon, 1993). These wild relatives presumably contain, among other things, potentially valuable genes for future disease or pest resistance. Erosion of genetic diversity in centers of diversity is already occurring at an alarming rate (Fowler and Mooney, 1990).

At the community level, transgenes which improve the plant's fitness could lead to an ecological release (Schmitt and Linder, 1994). Such a release could lead to an expansion of the species within the community or expansion into a new ecological range. These expansions have the potential of mirroring invasions of non-native introduced pests (Mooney and Drake, 1986; 1990). Invasions of non-native pests have caused among other things, species displacement, interruption and redirection of successional change, and changes in abiotic conditions such as moisture and salinity levels and soil nutrient and biotic composition. Changes such as these have the potential of fomenting a cascade of similar changes in an ecosystem.

It is now quite evident that the risk of engineered genes escaping into natural populations is real. Of the worlds 20 major crop species, all, with the exception of soybean, *Glycine max*, peanut, *Arachis hypogea*, coffee, *Coffea arabica*, chick pea, *Cicer arietinum*, and sweet potato, *Ipomoea batatas*, have been found to naturally

produce hybrids with their wild relatives (Hancock et al. manuscript in prep.; Ellstrand et al., manuscript in prep.).

Whether or not transgenic crops will be grown in close enough proximity to their wild relatives to allow pollinations/hybridizations to occur is probably also a foregone conclusion. Although there are only ten crop species with compatible wild relatives in the U.S., compatible wild relatives of all our crop plants are found somewhere (Hancock, et al., manuscript in prep). It is unlikely that the use of transgenic crops will be restricted to areas where the crop and its wild relatives do not come in contact. In some of the areas of the world where the perceived need for transgenics is highest they are most likely to come in contact with wild relatives (Hodgson, 1992; Gershon, 1992; Miller et al., 1995). In fact, transgenic crops are being commercialized most aggresively in just some of these regions (Moffat, 1994). Examples include Mexico, the center of diversity for maize, Central and South America, center of diversity for crops such as squash, potato, tomato and peanut, and Southeast Asia, center of diversity for rice, banana, citrus and sugar cane.

Given the near certainty that domesticated genes will escape if fertile transgenics are planted in close proximity to their wild congeners, several mechanisms for containing the pollen-mediated spread of transgenes to their wild relatives have been proposed. These include: 1) Isolating the transgenic crop by distance from its wild relatives, 2) Using barrier or guard rows to trap or intercept transgenic pollen from leaving the plot, or 3) Genetically isolating the crop through the use of male sterility or pollen-lethal genes (Ellstrand, 1988; Ellstrand and Hoffman, 1990; Kareiva et al., 1994). Tests of these mechanisms have been performed; isolation by distance,

(Manasse, 1992; Morris et al., 1994), barrier or guard rows, (Tynan et al., 1990; Umbeck et al., 1991; Scheffler et al., 1993; Morris et al., 1994), male sterility, (Eber et al., 1994). Results from the tests of the isolation by distance mechanism suggest that barren zones or increased distance between blocks of plants might actually serve to increase the amount of gene movement out of the isolated plot rather than decrease it. Studies of the extent of gene movement in border plantings uniformly demonstrate that most pollen is deposited within a few meters of the source. However, none of these studies was designed to measure pollen movement beyond a contiguous border planting. Many such studies report a clustering of donor genes on the border edges suggesting that donor pollen may be moving beyond the border edge. Finally, in an experiment designed to evaluate the level of outcrossing of rapeseed, *Brassica napus*, to weedy relatives and the purity of F_1s produced in the presence of the weedy relatives when the cultivar was male sterile, it was found that spontaneous interspecific hybrids could be produced under natural conditions when the male sterile served as the female parent (Eber et al., 1994).

The specific goals of this dissertation were to address the following questions: 1) Can border rows be used as a means to effectively limit pollen movement, and 2) Are the pollen dispersal patterns of native and transgenes the same?

The initial chapter of the dissertation is a further characterization of the cucumber (*Cucumis sativus* L.) mutant, 'Wisconsin SMR-18 *bla*' (blunt leaf apex) facilitating its use as an isogenic recipient parent to study pollen movement. A new character, (truncate leaf base) associated with previously described characters provided a quick, reliable screen for the large numbers of seedlings evaluated in the experiments

described in Chapter two. Chapter two of this dissertation describes a set of experiments designed to test the efficacy of border rows as a means to restrict the pollen-mediated movement of transgenes out of crop plantings. The number of donor plants was varied in conjunction with varying border sizes to create four trap plant to donor plant ratios. The influence of these ratios on long distance gene movement to discontiguous satellite plots located 50 meters from the plot centers was evaluated. Although all the afore-mentioned studies, including our own, reported varying degrees of success in restricting the escape of engineered genes, protection was never complete. Taking into account the results of such tests, the consensus seems to be that regardless of the containment strategy employed, some genes will escape from agronomic scale plantings of transgenic crops.

With the knowledge that transgenes will escape, the next level of concern revolves around the nature of gene movement itself. What is the likelihood that transgenes will become established in natural populations? And what will be the rate of spread of these genes once they become established? Research dealing with actual transgene movement and establishment is only beginning to emerge (Crawley et al., 1993; McPartlan and Dale, 1994; Scheffler et al., 1993; Scheffler and Dale, 1994; Linder and Schmitt, 1994, 1995). Due to this limited amount of information generated by research with actual transgenic plants, much of the response to these issues has been based on a body of theoretical and empirical evidence accumulated from research done with non transgenic organisms (Andow, 1994; Crawley, 1987,1990; Darmency, 1994; Gliddon, 1994; Manasse and Kareiva, 1991; Mooney and Drake, 1990; Williamson, 1994).

The widespread use of this non-transgenic data base to respond to transgenic risk assessment issues is based on the assumption that transgenes will disperse in the same fashion as native genes. While for many this seems to be a reasonable assumption, the novel sources of some transgenes (Rissler and Mellon, 1993) and the fact that transgenes are not always predictably expressed in plants (Finnegan and McElroy, 1994) lead others to believe that any assumption concerning genetically engineered crops should be evaluated (Rissler and Mellon, 1993). Chapter three of this dissertation details a direct comparison of dispersal patterns for a transgene and native gene originating from the same donor. Additionally, we revisit the influence of border rows on long distance movement for the two types of genes.

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CHAPTER 1

CHARACTERIZATION OF THE BLUNT LEAF APEX (*bla*) TRAIT IN CUCUMBER

Abstract

A further characterization of the cucumber (*Cucumis sativus* L.) mutant, `Wisconsin SMR-18 *bla*' (blunt leaf apex), revealed a new character associated with the previously described leaf phenotype. The attachment of the blade to the petiole of *bla* plants is flat across as opposed to the cordate or indented attachment seen in the wild type `Wisconsin SMR-18' plants. The new character, truncate leaf base, was easier to score and becomes distinctive earlier in development than previously described leaf apex characters. It was expressed consistently in homozygous *bla* plants. Segregation analysis of 1159 F_2 seedlings arising from self pollinated `Wisconsin SMR-18'/Wisconsin SMR-18 *bla*' F_1 plants suggested that the leaf base and leaf apex characters were controlled by a single locus or two tightly linked ones with a maximum distance between the two of 0.03 cM. In a field study of growth and fitness characteristics, the two genotypes did not differ significantly for numbers of flowers or fruits. The similar flowering and fruiting characteristics along with the reliable, early occurring truncate character should make the two genotypes useful for pollination and gene movement studies.

Numerous genes have been described in cucumber (*Cucumis sativus* L.) (Pierce and Wehner, 1987, 1990). Among these, nine are associated with distinctive leaf morphologies (Vakalounakis, 1992). Leaf morphological mutations can be useful as markers for hybrid production, and for pollination, genetic, and linkage studies. We have been using the recessive *bla* (blunt leaf apex) mutant as a marker trait to monitor pollen mediated gene flow from donor (wild type) to recipient (*bla bla*) populations (Hokanson et al., 1994). The *bla* mutant was first described by Robinson (1987) as a recessive seedling marker trait that arose from a mutagenized 'Wisconsin SMR-18' cucumber population. Individuals expressing the trait were reported to have a rounded leaf apex, rather than the pointed leaf apex typical of wild type 'Wisconsin SMR-18'. Seeds of the *bla* mutant were originally provided by R. Robinson (NY Agric. Exp. Sta., Cornell University, Geneva, NY). Seed increases were performed by hand pollinations in the greenhouse and by field pollinations in bee-proof cages.

In the process of working with the mutant, we found that expression of the leaf apex trait was variable. The phenotypes observed for 'Wisconsin SMR-18' *bla* plants in the greenhouse ranged from leaves exhibiting extremely rounded leaf apices with reduced lobing and serration as originally described by Robinson (1987), to leaves with nearly pointed apices, and sufficient lobing and serration to blur the distinction between *bla* and wild type (Fig. 1A). The lobing and serration traits were quite variable. The wild type 'Wisconsin SMR-18' also could exhibit variable characteristics in the first true leaf. In a test seedling population, the apex of the first true leaf was blunt in 9 of 33 wild type 'Wisconsin SMR-18' plants.

Here we describe a second characteristic associated with the leaf genotype, a flat

Figure 1. *Bla* Phenotypes. A. Variation in leaf shape for first true leaves of 'Wisconsin SMR-18' *bla*. Arrows indicate a 'Wisconsin SMR-18' *bla* individual with a first leaf with an acute apex and truncate leaf base. B. Leaf phenotypes for 'Wisconsin SMR-18' (left) and 'Wisconsin SMR-18' *bla* (right). Note the indented or cordate leaf base for the 'Wisconsin SMR-18' leaf on the left and the truncate base for 'Wisconsin SMR-18' *bla* on the right. C. Progeny from an open-pollinated 'Wisconsin SMR-18' *bla* plant. White dots indicate wild type 'Wisconsin SMR-18' leaves originating from a heterozygous seedling (an outcrossing event). Note other leaves have the recessive truncate leaf base.

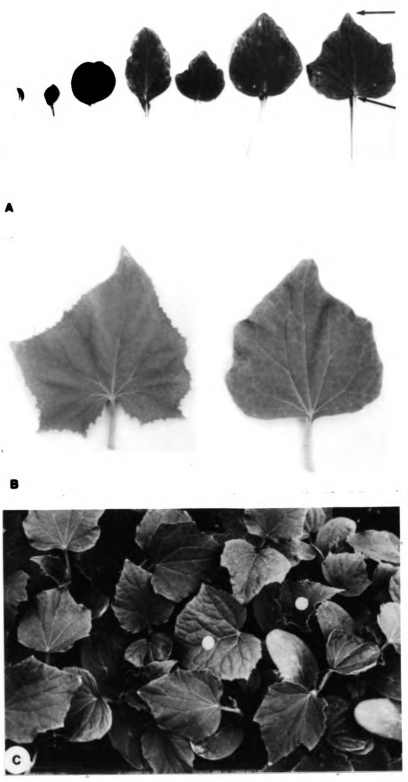

Figure 1.

or truncate leaf base. The attachment of the blade to the petiole of *bla* plants is flat across, rather than indented or cordate (Fig. 1A, B). This trait was readily observed in the second and all subsequent true leaves. The truncate leaf base was consistently expressed in homozygous *bla* plants (Fig. 1A). Regardless of leaf shape (i.e., apex, serration and lobing), if the first two leaves had truncate leaf bases, the plants always had the *bla* phenotype at maturity. Progeny of self-pollinated *bla* plants always exhibited the mutant phenotype. Although the first true leaves of some wild type 'Wisconsin SMR-18' plants (7 of 33) had a truncate leaf base, all subsequent leaves had cordate leaf bases and pointed leaf apices. Similarly, heterozygotes clearly exhibited the dominant, cordate leaf base (Fig. 1C).

To verify that the truncate leaf base trait was due to the presence of the *bla* mutation rather than a mutation at a separate locus, we self-pollinated flowers on 11 'Wisconsin SMR-18'/ 'Wisconsin SMR-18' *bla* F_1 plants. All 11 of these F_1 plants exhibited both the dominant cordate leaf base and wild type leaf apex. We evaluated 1159 seedlings arising from 16 different fruits from the 11 F_1 plants. Seedlings were assessed at the first, second, and third leaf stage for leaf base and leaf apex characters (Table 1). At the first leaf stage, approximately 10% of the wild type individuals resembled the mutant either for the shape of the leaf apex, the leaf base, or both; the apex was more variable than the base. When the second and third leaves were scored, both the leaf base and apex characters segregated in the expected 3:1 ratios for a recessive, single gene trait. Importantly, from the second leaf on, there was complete correlation between the two traits; no recombinants (blunt leaf apex associated with cordate leaf base or acute leaf apex with truncate base) were observed among the 1159 F_2 seedlings. These results

Table 1. Test for co-segregation of blunt leaf apex and cordate leaf base

Leaf position	Wild apex/ wild base	Wild apex/ truncate base	Blunt apex/ wild base	Blunt apex/ truncate base	Apex wild:blunt	Base wild:truncate
1st	769	4	61	325	773:386	820:329
					χ^2=42.9***,z	χ^2=7.09**,z
2nd	859	0	0	300	859:300	859:300
					χ^2=0.48	χ^2=0.48
3rd	861	0	0	298	861:298	861:298
					χ^2=0.31	χ^2=0.31

zChi-square test for an expected 3:1 segregation ratio for the individual characters blunt apex or truncate base.

,*Significant for one degree of freedom at the .01 and .001 levels respectively.

suggested that the leaf base and leaf apex characters were controlled by a single locus or two tightly linked ones (maximum distance 0.03 cM; product ratio method). Since expression of the *bla* and wild type phenotypes was more variable for the first leaf than for later leaves, it is important to score these traits no earlier than the second true leaf.

To further analyze the utility of this mutation, the *bla* mutant also was studied for growth and fitness characteristics in the field using a randomized complete block design with four replications. The two genotypes did not differ significantly for numbers of flowers or fruits (Table 2). Comparable flowering and fruiting characteristics between the two genotypes should allow for pollination and gene movement studies to be accomplished without bias due to diminished reproductive performance of one of the genotypes.

In summary, the occurrence of the truncate leaf base character increases the usefulness of the *bla* mutant for screening large seedling populations, especially in the early seedling stage. Although both characters (blunt apex and truncate base) are reliable in later stages of development, the leaf base character becomes distinctive and consistent sooner in development than does the leaf apex.

Table 2. Comparison of flowering and fruiting of 'Wisconsin SMR-18' and 'Wisconsin SMR-18' *bla* genotypes

Trait	'Wis. SMR-18'	'Wis. SMR-18' *bla*	MS_{trt} / MS_{Error}
Male flowers per plant[z]	49	42	35.9/61.0 ns
Total fruits per plot[y]	10	9	2.2/1.1 ns

Four replicate plots, five plants per plot, three central plants scored per plot. Plants were spaced 0.5 m within rows, 1.5 m between rows.

[z]Total number of open staminate flowers per plant (summed over three observation dates, August 6, 14 and 28, 1992). The two genotypes also did not differ significantly for number of female flowers.

[y]Total number of fruits (≥ 5 cm) on the vine on September 30, 1992.

[ns]Nonsignificant F-value for analysis of variance, $P > 0.05$.

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CHAPTER 2

EFFECT OF BORDER ROWS AND TRAP/DONOR RATIOS ON POLLEN-MEDIATED GENE MOVEMENT

Abstract

One frequently voiced concern associated with the field testing and agronomic scale release of transgenic crops is the potential for pollen-mediated escape of engineered genes into naturally occurring populations of wild relatives. Border rows have been commonly used for restricting the pollen-mediated escape of engineered genes in field tests. However, the efficacy of border rows for restricting such gene movement has been little studied. Isogenic lines of cucumber (*Cucumis sativus* L.) differing for a seedling marker trait, blunt leaf apex (*bla*) were planted in various trap plant to donor plant ratios to test border rows as a means to control pollen-mediated gene movement out of plantings of insect pollinated crops. All treatments had donor plants, (Wisconsin SMR-18 with the wild type leaf shape) in the center of the plot. Three of the four treatments had border plantings of recipients, ('SMR-18*bla*') surrounding the donors. For each of the plots, groups of four recipient plants (satellites) were planted fifty meters from the plot center in eight directions. Progeny of the recipient plants from the satellites and borders were screened to determine the percentage of outcrossing as measured by occurrence of the dominant phenotype. Gene movement within the plot borders assumed a leptokurtic distribution. At each distance from the plot center, there was more gene movement in plots with $2m^2$ donors than $1m^2$ donors. Long distance gene movement to the satellites increased significantly as the trap/donor ratio decreased. These results suggest that border rows

might serve to control the movement of transgenic pollen in small experimental plots. However, variability in amounts of gene movement to individual satellites within treatments (ranging from 0-38%) suggests environmental variables might render predictions concerning gene flow levels and containment strategies quite difficult. Moreover, to achieve the trap/donor ratios of the most protective treatment in these experiments on a commercial scale would in all likelihood be economically infeasible.

Introduction

Pollen mediated escape of engineered genes into the environment frequently has been mentioned as a potential risk associated with the large-scale release of genetically engineered transgenic crops (Colwell et al., 1985; Ellstrand, 1988; Ellstrand and Hoffman, 1990; Dale, 1992; Raybould and Gray, 1994; Rogers and Parks, 1995). A commonly described scenario involves the movement of engineered genes into wild or weedy relatives. Crop/weed hybridizations have been documented and can involve significant amounts of long distance gene movement. For example, Klinger et al. (1991,1992) measured levels of hybridization between cultivated and wild radish, *Raphanus sativus* L. at distances up to 1,000 m. Kirkpatrick and Wilson (1988) reported hybridizations in both directions between cultivated *Cucurbita pepo* and naturally occurring Texas gourd *Cucurbita texana* at distances of 1,300 m. Wilson and Manhart (1993) found high rates of hybridization between cultivated *Chenopodium quinoa* (Andean grain chenopod) and a North American wild relative *C. berlandieri* at 500 m from the cultivated plot.

With regard to the concerns associated with transgenic plants, several schemes

have been proposed to restrict pollen mediated escape of engineered genes. These include: 1) isolating the genetically engineered crop by distance from wild relatives, 2) surrounding the genetically engineered crop with rows of non-engineered pollen trap plants, and 3) genetic isolation of the transgenic crop using mechanisms such as male sterility, (if the fruit or seed is not the product of commerce), or linking the engineered gene to a pollen lethal gene (Ellstrand and Hoffman, 1990; Kareiva et al., 1994). To date, isolation by distance, and the use of border rows of pollen trap plants have been the most widely utilized methods to satisfy USDA requirements for field testing transgenic plants (Wrubel et al., 1992).

The use of isolation by distance and border rows to limit the spread of transgenic pollen stems from the historical precedent of using these methods to ensure genetic purity in seed multiplication plots (George, 1985; Kelly, 1988). While these methods have been used successfully to prevent undesirable pollen from moving into seed multiplication plots, the question remains as to whether they can control the movement of genes out of plots. Several recent studies have suggested that isolation by distance may be an unreliable method to control pollen mediated gene escape. Manasse (1992) found that increasing isolation distances from 0.5m to 4m between individuals or groups of *Brassica campestris* also increased mean gene flow. When Morris et al. (1994) compared the use of border rows to barren zones as a means to control the spread of transgenes from *Brassica napus*, they concluded that the use of barren zones might actually increase the amounts of gene movement over that which would be expected if the same space were planted with a crop. They also found that gene movement levels differed between locations, signaling the key role played by

environmental variables in gene movement.

Border rows have been used as a method to maintain genetic purity in wind pollinated seed crops such as beet, *Beta vulgaris* (Dark, 1971) corn, *Zea mays* (Kelly, 1988) and some insect pollinated crops such as cotton *Gossypium sp.* (Green and Jones, 1953). Isolation results from either a physical blocking of foreign pollen by the height and density characteristics of the border crop or by diluting the foreign pollen with "non-polluting" border row pollen. A simple extension of this thinking leads to the idea that these methods might also serve to keep pollen from moving out of a bordered plot.

The few studies testing border rows as a means to control movement of genes out of a plot have focused on gene movement into contiguous border plantings. Tynan et al. (1990) reported low levels of gene movement from transgenic *Solanum tuberosum* into wild-type potatoes planted within the transgenic trial and in contiguous border plantings of the wild-type. Values ranged from approximately 1% among the inter-planted wild and engineered types to 0.05% at 3-4.5m in the border; no movement was recorded beyond this distance. Scheffler et al. (1993) measured movement of a transgene from a 9 m circular center plot of engineered *Brassica napus* into a contiguous 70 m^2 border of non transgenic *B. napus*. They found negligible amounts of movement of the marker gene beyond 6 m, (less than 0.03%) and no movement beyond 36 m. Umbeck et al. (1991) reported a "consistent and significant" reduction in outcrossing into a 25 m wide border as the distance from the donors increased. Outcrossing ranged from nearly 5% at the border of the donors to less than 1% at 25 m.

Although these studies indicate that most pollen is distributed within a short distance from the donors and should therefore be trapped by borders, they do not address the question of gene movement into non-contiguous plantings which would more closely mirror the situation for patches of wild relatives. When Handel (1982) used a dominant morphological marker to measure gene movement within an 18 m^2 plot of cultivated melons (*Cucumis melo)*, he found that gene movement was asymmetrical with occasional large numbers of dominant seedlings arising from fruits at the edge of the plot. In similar experiments with cucumber (*Cucumis sativus*), he found clumping of genes on the edge of 25 m^2 and 16 by 12 m plots (Handel, 1983). The aggregation of genes on plot edges may be due to an ecotonal effect or they may suggest that pollinators carry marked pollen out of the plot.

While all the above studies have generated important information on patterns of gene flow, no controlled experiments have been designed to test the effectiveness of border rows in preventing long distance gene movement to discontiguous plots. Also, no studies have directly tested the effects of varying relative donor plant to trap plant ratios on gene movement. In this study we investigate varying donor plant to trap plant ratios and predict that as trap/donor ratios decrease, more pollen would escape due to an overloading of the trap plants with donor pollen. In the experiments described herein, we used a morphologically marked cucumber genotype to address the following questions: 1) What is the frequency and pattern of gene movement into the contiguous border, 2) What is the effect of varying relative donor plant to trap plant ratios on the rate of gene movement, and 3) Can border rows effectively limit long distance movement of donor genes as measured by discontiguous satellite plots?

Materials and Methods

Plant material. The monoecious isogenic cucumber (*Cucumis sativus* L.) lines Wisconsin SMR-18 and SMR-18 *bla* (blunt leaf apex) were used to monitor pollen movement. Cucumber is a predominantly outcrossing crop, 23-77% in the field, (Wehner & Jenkins, 1985), that is pollinated primarily by honeybees, *Apis mellifera* (Free, 1993). SMR-18 *bla* is a recessive mutation which arose from a mutagenized Wisconsin SMR-18 population (Robinson, 1987). Individuals expressing the *bla* trait have reduced lobing and serration, a rounded leaf apex rather than the pointed leaf apex typical of wild type Wisconsin SMR-18, and a flat leaf attachment as compared to the indented attachment of Wisconsin SMR-18. The leaf attachment character serves as a reliable, readily scorable seedling marker trait (Hokanson et al., 1995). Wisconsin SMR-18 and SMR-18 *bla* were shown to have comparable flowering and fruiting characteristics (Hokanson et al., 1995). Wisconsin SMR-18 seed was purchased from Agway Inc. (Syracuse, NY). SMR-18 *bla* seed was originally provided by R. Robinson (New York State Agriculture Experiment Station, Cornell University, Geneva, NY) and subsequently field multiplied in bee-proof cages.

Field experiments and greenhouse screening. The dominant, wild type genotype Wisconsin SMR-18 was used as the pollen donor, and the recessive SMR-18 *bla* as a pollen trap or recipient. The overall plot design is depicted in Figure 1. Gene movement was detected by screening for dominant type seedlings among seedlings originating from recessive plants in the plot borders and satellites.

Four treatments varying in ratio of trap to donor plants were utilized to test the

Figure 1. A. Overall field plot design used in 1992 and 1993 experiments. All 12 plots regardless of design were surrounded by eight satellite plots 50 meters distant, each containing four recessive SMR-18 *bla* plants. B-E. Four treatments tested in these experiments depicted from highest to lowest trap to donor ratio. B. $1m^2$ of SMR-18 donors surrounded by a $400m^2$ border of recessive SMR-18 *bla* plants (ratio 131.1). C. $1m^2$ of donors surrounded by a $100m^2$ border of recessive recipients (ratio 34.0). D. $4m^2$ of donors surrounded by a $100m^2$ border of recessive recipients (ratio 11.6). E. $1m^2$ of SMR-18 donors with no surrounding border plants (borderless)(ratio 0.0). At harvest borders were subdivided into $1m^2$ subplots and 2-3 fruit were collected from each subplot (see also Fig. 3). ▲ Location of bee hives on the edge of the donor plots.

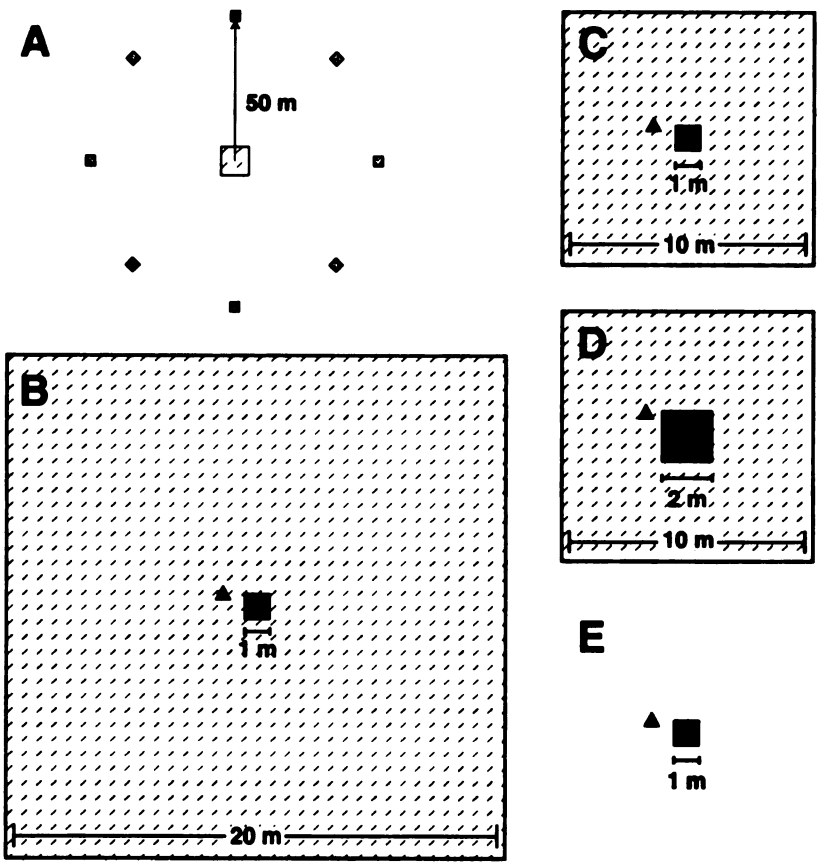

Figure 1.

efficacy of border rows: 1) 1 m^2 of donor plants surrounded by 400 m^2 of border plants, 2) 1 m^2 donors with a 100 m^2 border, 3) 4 m^2 donors with a 100 m^2 border, and 4) 1 m^2 donors with no border (Table 1). The 400 m^2 border plots were comprised of 29 rows with 41 plants per row; 9 donors were placed in the middle of the center 3 rows. The 100 m^2 border plots had 15 rows with 21 plants per row with 9 or 25 donors in the middle of the center 3 or 5 rows respectively. The borderless plot contained 9 donor plants surrounded by a 100 m^2 cultivated, unplanted area. All of the donor populations, regardless of treatment, were surrounded by eight satellite plots which were located 50 m from the plot center (Fig. 1). Each satellite plot, which measured approximately 1.2 m x 1.2 m, contained four cucumber plants with the recessive *bla* phenotype.

All four treatments were tested in the summer of 1992, each replicated in two locations (Table 1). In the summer of 1993, two of these treatments were repeated at two locations, the 100 m^2 border with 1 m^2 donors and the borderless plot with 1 m^2 donors (Table 1). Planting dates were June 15 and 16 in 1992, and June 16 in 1993.

All plots were isolated by at least 1500 m from any other cucumber plantings. The plots were maintained in a manner similar to commercial plantings (Motes, 1977). During preparative cultivation, 16·16·16 fertilizer was incorporated into the soil at a rate of 452.5 kg per hectare. Approximately six weeks later, the rows were sidedressed with nitrogen at a rate of 45 kg per hectare. All plots were hand cultivated until a full canopy developed. About 1.3 cm of water per week was applied to the plots in the absence of adequate rain. When the plants were in full flower and female flowers were opening, (August 20 in 1992 and July 23 in 1993), one bee hive

Table 1. Trap/donor ratio for each of the four treatments with sites and locations for the replications of each in 1992 and 1993.

Treatment	Trap/donor ratio	Site	Abbreviation	Location	Year
1 m^2 donor,	131.1	Lux Arbor Reserve	Lux	KBS[2]	92
400 m^2 border	1180/9[1]	Botany/Plant Pathology Farm	Bot	MSU[3]	92
1 m^2 donor,	34.0	Long Term Ecological Research Site	LTER-1	KBS[2]	92
100 m^2 border	306/9	Muck Farm	Muck	MSU[3]	92
		Long term Ecological Research Site	LTER-2	KBS[2]	93
		Botany/Plant Pathology Farm	Bot	MSU[3]	93
4 m^2 donor,	11.6	Kellogg Farms	Kell-1	KBS[2]	92
10 m^2 border	290/25	Crop and Soil Science Farm	Crops	MSU[3]	92
1 m^2 donor,	0.0	Kellogg Dairy Farm	Dairy	KBS[2]	92
No border	0/9	Inland Lakes Research Area	Ponds	MSU[3]	92
		Kellogg Farms-2	Kell-2	KBS[2]	93
		Palmerly Farm	Palm	MSU[3]	93

[1]Number of plants in the border/number of plants in donor plot.
[2]KBS, Kellogg Biological Station, Kalamazoo County, MI.
[3]MSU, Michigan State University Farm, Ingham County, MI.

containing approximately 35,000 honeybees, *Apis mellifera* was placed on the edge of the donor plants with the hive opening facing southeast (Fig. 1). The hives were removed prior to fruit harvest (September 18 and August 31 respectively).

To evaluate long distance gene movement, fruits were harvested from the individual satellites, and stored in plastic bags at 0-5°C until seeds were extracted. The plots were harvested on October 5 and 6 in 1992 and September 8 and 15 in 1993. Seeds were extracted from each fruit, air dried, bulked by satellite in paper seed envelopes, and stored at 10°C and 25% relative humidity.

The seeds were germinated and scored in a greenhouse at Michigan State University, East Lansing, MI, beginning in January of 1993. The seeds were planted 75 to a tray (15 seeds per row, 5 rows per tray) in 56 cm x 28 cm plastic seedling trays. The seedlings were grown in a soil mix of 1 sphagnum peat perlite mix (Baccto Professional Planting Mix, Michigan Peat Co., Houston, TX): 1 sterilized sandy loam. From mid-November through mid-April, the seedlings were grown under artificial light ranging in intensity from 258 $\mu mol \cdot s^{-1} \cdot m^2$ photosynthetically active radiation (PAR) on an overcast day to 530 $\mu mol \cdot s^{-1} \cdot m^2$ PAR on a sunny day.

The plot borders were divided into 1 m^2 subplots to evaluate short distance gene movement. Fruits were harvested from each subplot on the same dates as the satellites. Fruit and seed were processed as described above.

Data analysis. Both short and long distance gene movement was expressed as the percentage of dominant type seedlings appearing among the seedlings germinated from recessive parent plants in the subplots and satellites respectively. To analyze short distance gene movement within the borders, the subplots were grouped according

to their distance from the plot center. To test the effect of increased donor plot size on percent outcrossing within the border planting, paired comparisons were made at each distance from the plot center (2 m - 7 m) for which direct comparisons could be made. Analyses were performed on data from pairs of plots (1 m^2/100 m^2 and 4 m^2/100 m^2) at the same location (KBS or MSU) in the same year. The data [12 pairs of 1 m^2 and 4 m^2 donor plots with 100 m^2 borders (6 distances, 2 locations)] were analyzed both by T-test of paired observations and Wilcoxin's signed rank test for paired observations. Both tests gave the same results. To evaluate long distance gene movement, mean percent gene movement into the satellites was plotted for both seasons as a function of trap/donor ratio. Data were analyzed by Spearman's coefficient of rank correlations and by regression analysis on arcsin linearized data as per Steele and Torrie (1960).

Results

Short distance gene movement. Gene movement within the borders assumed a leptokurtic distribution at all sites (Fig. 2). The highest percent gene movement, 74.0% and 91.0% were observed at the closest distances, one meter (34.0 trap/donor ratio) and two meters (11.6 trap/donor ratio) respectively. Values decreased rapidly with increasing distance from the plot center. Similar to what was observed by Handel (1982, 1983), individual plot maps revealed a few instances of high levels of outcrossing occuring at the plots' edge (Fig. 3). There were no consistent trends for overall gene distribution within the borders that could be attributed to the location of the hives.

Figure 2. Comparison of short distance gene movement (percent gene movement observed within the borders at one meter intervals from the plot center) for plots with $1m^2$, ▲, or $4m^2$, • donor plots. One square meter data are the mean of all plots with $1m^2$ donors in 1992 and 1993, $4m^2$ data are the mean of the two $4m^2/100m^2$ plots in 1992. Data for paired comparisons came from $1m^2$ and $4m^2$ donor plots with $100m^2$ borders at two locations in 1992.

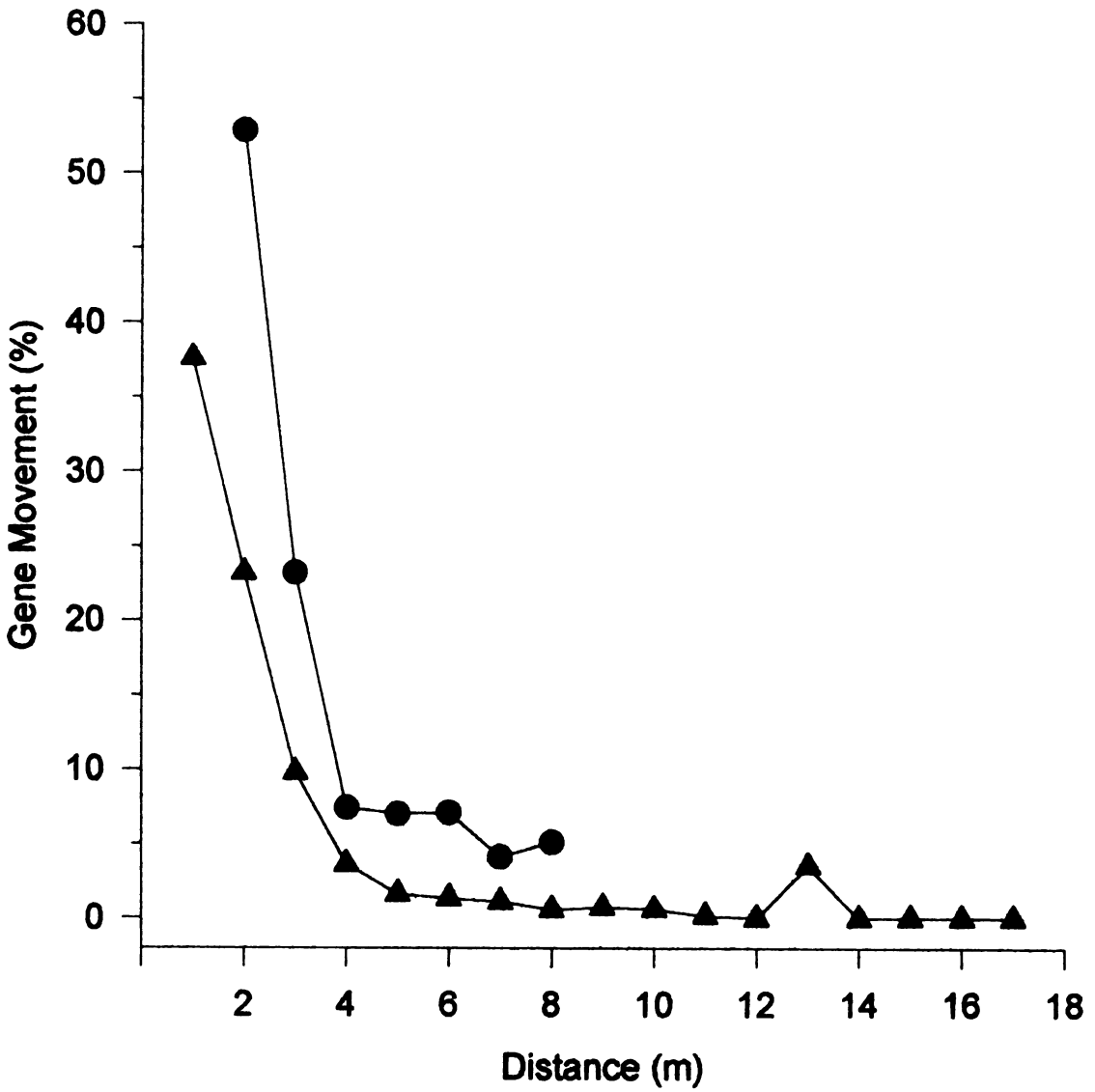

Figure 2.

Figure 3. Representative example of gene movement into the border subplots (plotted as % wild type seedlings among all seedlings in each 1 m^2 subplot) for the 1992 Kell-1 plot. Each square represents a $1m^2$ subplot.

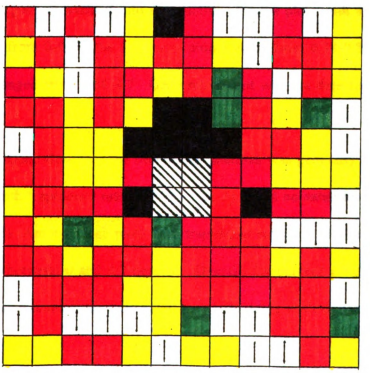
% Wild type seedlings
Not sampled
0%
1-10%
11-20%
21-30%
>31%
Donor plants

Figure 3.

Movement from the 1 m^2 and 4 m^2 donor plots (34.0 and 11.6 trap/donor ratios respectively) had similar distribution patterns (Fig. 2). However, there was significantly more gene movement into the plot borders from the 4 m^2 donor plots than the 1 m^2 donor plots ($p \leq 0.005$; T-test or Wilcoxin sign rank test of 4 m^2 and 1 m^2 donor plot data paired by distance from the plot center).

Long distance gene movement. As was the case with short distance gene movement, there was no apparent association between hive placement and patterns of long distance gene dispersal to the satellite plots. Gene flow to the satellites was highest in the absence of border/trap plantings (Fig. 4; Table 2). In general, long distance gene movement into the satellites decreased significantly as the trap/donor ratio increased (Table 2; Fig. 4, linearized regression $y = 8.11 - 5.23x$, $df = 10$; $r^2 = 0.671$, $p \leq 0.05$). The Spearman's coefficient of rank correlation was $r_s = 0.906$. The percent gene movement into the satellites for the plots with a trap /donor ratio of 34.0 ranged from 0-0.18, the 11.6 ratio plots ranged from 0.38-0.83, while the borderless plots ranged from 0.68-4.7. Gene movement was not detected for the 131.1 ratio plots.

Gene flow into the individual satellites was generally low (Table 3). All of the plots with trap/donor ratios of 131.1 or 34.0 had no satellites with gene movement over 1%. Among those plots with trap/donor ratios of 11.6 and 0, values generally ranged between 0 and 4.7 percent. Overall, the percent gene movement was evenly distributed among the eight satellites surrounding each donor plot (Table 3).

Figure 4. Mean values (± standard errors) for long distance gene movement (% of total seedlings with the dominant marker trait) to satellite plots for the four treatments tested in 1992 and 1993, plotted as a function of the trap/donor ratio for the treatment.

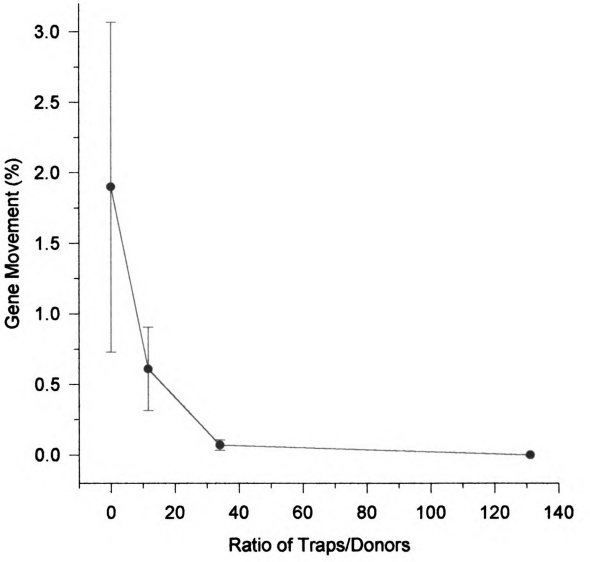
Gene Movement (%)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
80
100
120
140
Ratio of Traps/Donors

Figure 4.

Table 2. Mean long distance gene movement to the satellite plots for the four treatments in 1992 and 1993.

Treatment	Trap/donor	Site	Year	# Screened	% Gene Movement[1]
1 m^2 donor	131.1	Lux	92	637	0.00
400 m^2 border		Bot	92	574	0.00
1 m^2 donor	34.0	LTER-1	92	1144	0.18
100 m^2 border		Muck	92	161	0.00
		Bot	93	2372	0.04
		LTER-2	93	1588	0.06
4 m^2 donor	11.6	Kell-1	92	781	0.38
100 m^2 border		Crops	92	465	0.83
1 m^2 no border	0.0	Dairy	92	1030	0.68
		Ponds	92	403	4.70
		Kell-2	93	1854	1.42
		Palm	93	1971	0.70

[1]Mean values from eight satellites at each location

Table 3. Long distance gene movement (expressed as the % wild type seedlings in each satellite) to the eight individual satellites within each of the twelve plots tested in the summers of 1992 and 1993.

Location	Year	Trap/donor ratio	1	2	3	4	5	6	7	8	Mean
Lux	1992	131.1	(-)[1]	(-)	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Bot	1992	131.1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
LTER-1	1992	34.0	0.00	0.00	0.00	0.00	0.91	0.51	0.00	0.00	0.18
Muck	1992	34.0	0.00	(-)	0.00	0.00	(-)	(-)	0.00	0.00	0.00
Bot	1993	34.0	0.31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.04
LTER-2	1993	34.0	0.00	0.00	0.48	0.00	0.00	0.00	0.00	0.00	0.06
Kell-1	1992	11.6	0.93	0.00	(-)	0.00	1.10	0.00	0.00	0.00	0.29
Crops	1992	11.6	4.70	0.00	0.00	0.99	0.00	0.00	0.00	0.91	0.83
Dairy	1992	0.0	1.50	0.00	0.00	0.00	0.00	0.00	0.55	3.40	0.68
Ponds	1992	0.0	0.00	0.00	0.00	37.6	0.00	0.00	0.00	0.00	4.70
Kell-2	1993	0.0	0.00	(-)	1.00	1.70	0.36	1.50	0.97	4.30	1.40
Palm	1993	0.0	0.42	0.96	1.70	0.82	1.20	0.00	0.46	0.00	0.70

[1]No viable seeds

The only exception was the borderless Ponds plot with a mean value for long distance gene movement to the satellites of 4.7%. However, only one of the eight Ponds satellites actually received any gene flow, nearly 38%.

Discussion

Our results suggest that in small research plots, border rows might serve to effectively reduce the pollen mediated spread of engineered genes. We found that increasing the trap/donor ratios within the plots significantly reduced the long distance movement of genes into satellite plots. We did not detect any pollen movement outside the borders with a trap recipient to donor ratio of 131.1, and even the least protective border treatment, with a trap/donor ratio of 11.6, allowed an average of only 0.75% long distance gene movement.

While such borders can probably be used to minimize gene flow within small research plots, it would be difficult to limit gene flow out of agronomic-scale plantings. Our data indicate that borders could be "swamped" unless they were larger than the transgenic fields themselves. We found that increasing the number of donors within the 100 m^2 border, i.e. lowering the trap/donor ratio, had two effects. First, the amount of gene movement into the border (short distance movement), was greater at all distances from the plot center for the 4 m^2 as compared to the 1 m^2 donor plots. Secondly, gene movement to the satellite plots, (long distance movement), increased as the trap/donor ratio decreased. At our most effective trap/recipient ratio, over 100 acres of non-transgenic trap plants would need to be planted for each acre of transgenic plants to prevent gene escape.

Environmental variation also makes it difficult to develop strategies for containing transgenes in agronomic settings. For example, we found that long distance movement to individual satellites within the plots was generally low and fairly uniform, (0-4.7%), however one satellite within the Ponds borderless plot received 38%. This satellite had several small lakes (ponds) located approximately 0.25 miles beyond it. Perhaps these lakes, being the primary water source in the area structured bee movements creating a directionality to their food foraging leaving open the possibility that a single environmental variation may have resulted in a major shift in gene movement.

When large numbers of transgenic crop plants are deployed, the sheer volume of crop genes moving into surrounding wild populations could ensure their persistence in hybrids. One of the axioms of invasion biology is that invaders are more likely to succeed when they have a large founding population (Mooney and Drake, 1990). The Ponds satellite with 38% gene flow is a good example of what has been referred to as a "low frequency, large magnitude event".

It is often argued that some of the genes proposed for incorporation into crop genomes via biotechnology, such as those conferring tolerance to salinity, drought or cold could persist in crop/wild hybrids by increasing the selective advantage of the plants in which they reside. However, selective advantage or increased fitness aside, in those instances where large numbers of crop/wild seeds are produced through a flooding of domesticated pollen into wild populations, a few hybrids might survive (Glidden, 1994). As previously discussed, several successful crop/weed hybridizations have been documented in situations where the crop and its weedy relative co-occur;

(*Daucus carota* L., Wijnheijmer et al., 1989; *Oryza sativa*, Langevin et al., 1990; *Zea mays*, Doebley, 1990; *Beta vulgaris* L., Santoni and Berville, 1992; Boudry et al., 1993; *Setaria italica*, Till-Bottrand et al., 1992). Moreover, when Klinger and Ellstrand (1994) created crop/weed hybrids in *Raphanus sativus* L., they found a 15% increase in fruit and seed production as compared to wild siblings, with no reduction in the other fitness characters measured; time to first flower, early fruit production, average seeds per fruit, and average seed mass. Thus, in at least some instances, crop/weed hybrids can actually have a higher level of fitness than their wild siblings.

In conclusion, while the use of border rows can reduce the extent of gene movement out of a small test plot, use of border rows as a containment strategy in agronomic-scale plantings is of dubious value. The numbers of non-transgenics that must be planted to significantly reduce escape is not agronomically feasible. Moreover, even in the most protective schemes, environmental variation can result in substantial levels of isolated gene flow. In making decisions about the large scale release of transgenic crops, it may be more prudent to consider the nature of the gene itself and the crop in which it is to be deployed, rather than whether or not it can be contained.

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CHAPTER 3

COMPARISON OF DISPERSAL PATTERNS OF A TRANSGENE AND A NATIVE MARKER GENE ORIGINATING FROM THE SAME DONOR

Abstract

Despite full commercial approval of twelve transgenic crops in the U.S. in 1995, concern is still being expressed regarding potential risks associated with transgenic crops. A commonly voiced concern relates to the pollen-mediated escape of engineered genes into populations of crop wild relatives. To address this concern the scientific community has turned to a rich body of literature on pollen dispersal that has been generated on non-transgenic organisms. However, the utilization of this information requires the assumption that the pollen dispersal patterns of native and transgenes will be the same. To test the validity of this assumption, we evaluated levels of pollen-mediated gene movement from melon plants (*Cucumis melo*) expressing dominant native and engineered marker genes into a surrounding contiguous border and into discontiguous satellite plots of recessive non-transgenic melon plants. Long distance dispersal patterns of the native and transgene in these experiments was identical. At no time did we observe movement of one of the genes without the other. Long distance gene movement from plots with contiguous borders was significantly reduced relative to borderless control plots. Dispersal patterns of the two genes into the plot borders was nearly identical. Of the nearly 4600 seedlings screened for both morphological (presence of green cotyledons) and transgene movement (presence of NPT II protein by ELISA), in no case was the NPT gene observed in the absence of dominant Vir trait. However, 39 seedlings were green but

did not express NPT II as measured by ELISA. PCR analysis revealed 27 of these 39 NPT II ELISA$^-$ plants to contain the NPT II gene implicating transgene inactivation as the cause of the NPT II ELISA$^-$ seedlings. Segregation data suggested that the remaining 12 NPT II ELISA$^-$ plants were most likely the progeny of a heterozygous green, non-transgenic mother inadvertantly planted in the border.

Introduction

The commercialization of transgenic crops is now a reality. Twelve transgenic crop/gene combinations have now been given full commercial approval and more are soon to follow (Purrington and Bergelson, 1995; Rissler and Mellon, 1995). Despite the approval granted for these transgenic crops, concern is still being expressed regarding the potential risks associated with the commercialization of genetically engineered crops.

One of the most commonly raised concerns relates to the potential for engineered genes to move via pollen into populations of crop wild relatives (Colwell et al., 1985; Ellstrand, 1988; Ellstrand and Hoffman, 1990; Dale, 1992; Raybould and Gray, 1994). Although "escapes" from experimental-sized plantings can be minimized by any of a number of mechanisims including: isolation by distance, enclosure by border rows of pollen trap plants, and/or the use of genetic isolation methods (Ellstrand, 1988; Ellstrand and Hoffman, 1990; Kareiva et al., 1994), some level of gene escape is virtually inevitable in commercial scale plantings (Kareiva et al., 1994; Manasse, 1992; Hokanson et al., 1996).

Given the near certainty that transgenes will escape, concern now centers on

the establishment and spread of transgenes in natural populations. Direct measurements of actual transgene establishment and spread is quite limited (Crawley et al., 1993; Bergelson, 1994; McPartlan and Dale, 1994; Scheffler and Dale, 1993; Linder and Schmitt, 1994, 1995). This is due to the comparatively short time that transgenes have been available and to regulations limiting the scale and duration of field experiments utilizing them. As a result, much of the body of evidence amassed to respond to risk assessment issues has been based on a rich body of theoretical and empirical research on gene dispersal completed with non-transgenic organisms (Andow, 1994; Crawley, 1987, 1990; Darmency, 1994; Gliddon, 1994; Manasse and Kareiva, 1991; Mooney and Drake, 1990; Williamson, 1994).

An unspoken assumption underlies the use of information gained from non-transgenic organisms in making predictions concerning the behavior of transgenes. The assumption is that transgenes will move, in a manner analogous to that of native genes, i.e., in accordance with the rules of transmission genetics as we understand them today. Once a gene becomes integrated into the chromosome, regardless of the means, it should be transmitted between individuals in the same fashion as any other gene. While this assumption is probably valid, it remains untested.

Evidence is now accumulating that transgenes in plants are not always expressed in a predictable fashion (Finnegan and McElroy, 1994 and references therin). These abnormalities, known collectively as transgene inactivation, have produced some unexpected phenotypes. In fact, the various phenotypic effects associated with these inactivation events at times appear to result in abberant transmission patterns. Given some of the unique sources and the sometimes

unpredictable expression patterns of the genes, it is not so surprising that there are concerns that transgenes could segregate and transmit in an abberant manner (Rissler and Mellon, 1993). Since no direct tests comparing the movement of native and transgenes has been reported, no contention to the contrary can be offered.

In these experiments we address the issue of whether native and transgenes have the same pollen-mediated gene dispersal patterns. We compared the levels of gene movement from melon plants, *Cucumis melo*, expressing both a dominant morphological marker gene and a transgene, into a surrounding border of non-transgenic melons which did not contain the marker genes. Additionally, we extended an ongoing series of experiments (Hokanson et al., 1996) that test the efficacy of border rows as a means to control long distance gene movement.

Methods and Materials

Plant Material Two lines of melon (*Cucumis melo* L.) were used in this study. Transgenic plants expressing the NPT II (neomycin phosphotransferase) gene and carrying a dominant morphological marker for green cotyledons or non-virescence (V) were utilized as donor plants in these experiments. These donor plants (line ZYCP 30) were R_2 progeny of previously described transgenic plants (Fang and Grumet, 1993). Non-transgenic plants homozygous for the recessive virescent (v) trait were used as recipients. The monoecious virescent mutant (C879-J2), provided by Dr. Perry Nugent (U.S. Vegetable Laboratory, USDA, Charleston, SC), has been described previously (Nugent, 1987). Pleiotropic effects of the mutation include yellow cotyledons which turn green in approximately one week and cream colored flowers

that fade to white. The virescent mutant has been reported to exhibit 49% outcrossing in the field (Nugent, 1987). The two genes were tested for independent segregation (Table 1). Progeny segregating from a testcross of a heterozygous green, NPT II positive plant x two virescent NPT II negative plants fit a 1:1:1:1 segregation model.

Field Experiments The overall field plot design used in these experiments is depicted in Figure 1A. All donor (dominant, non-virescent, transgenic plants expressing the NPT II gene) plots were surrounded by eight recipient (recessive, non-transgenic, virescent plants) satellite plots located 50m distant. Each satellite plot, which measured approximately 1.2m x 1.2m, contained four recipient plants. Two treatments were used in these experiments (Fig.1). One treatment consisted of $1m^2$ of donor plants surrounded by $100m^2$ of recipient plants (Fig. 1B). This plot had 11 rows with 17 plants per row. Nine donor plants were placed in the center of the middle three rows. The other treatment had $1m^2$ or nine donor plants encircled by a $100m^2$ barren zone (Fig. 1C). Both treatments were replicated at two locations in the summers of 1993 and 1994 (Table 2). The plots were established on June 23, 24 and 25 in 1993 and June 9, 10 and 14 in 1994. The data presented are from the 1994 season. Although similar trends were seen in the 1993 data, the summer of 1993 was a poor growing year for melons resulting in low amounts of poor quality seed.

In order to prevent pollen contamination from other melon plants, all plots were isolated from other melon plantings by at least 1500m. Plots were maintained in a manner approaching commerical practices. Prior to planting, 12·12·12 fertilizer was incorporated into the soil at a rate of 453.5 kilograms per hectare. Roughly six weeks later, plants were sidedressed with 20.5 kilograms per hectare of nitrogen. Weeds

Table 1. Test for independent segregation of virescence (vir) and neomycin phosphotransferase (NPT II)

Classes	Observed	Expected	χ^2
Yellow NPT II ELISA$^-$	45	45.5	0.196^{ns}
Yellow NPT II ELISA$^+$	45	45.5	
Green NPT II ELISA$^-$	44	45.5	
Green NPT II ELISA$^+$	48	45.5	

^{ns}Not significantly different from the expected 1:1:1:1 ratio.

Figure 1. A. Overall field plot design. Each main plot (center square), was surrounded by eight satellite plots (shaded), located 50 meters from the plot center. Each satellite plot contained four non-transgenic recessive virescent melon plants. B and C. Expanded view of center plots. B. The bordered plot contained $1m^2$ of transgenic non-virescent donor plants (small center square), surrounded by a $100m^2$ border of non-transgenic recessive virescent melon plants. C. Borderless center plot contained $1m^2$ of transgenic non-virescent donor plants. ▲ Location of bee hive on the edge of the donor plots.

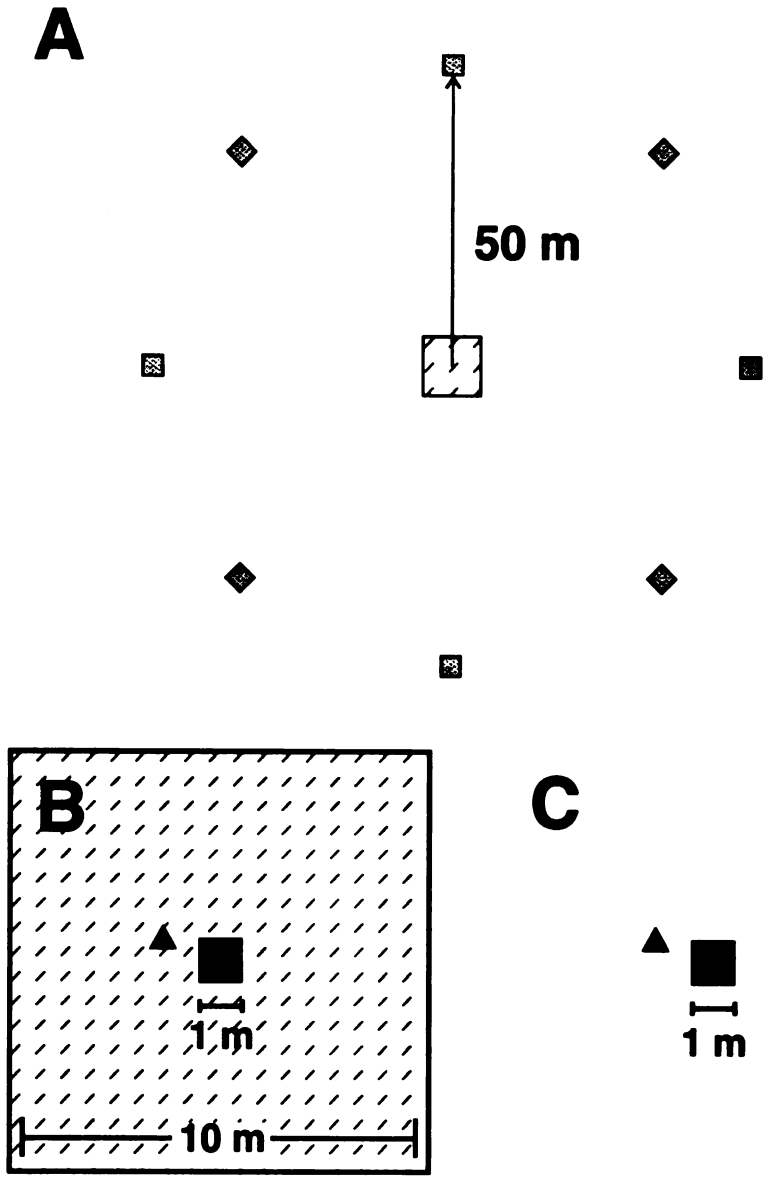

Figure 1.

Table 2. Trap/donor ratios and locations for each of the treatments tested in 1994.

Treatment	Trap/donor ratio	Site/location	Abbreviation
1 m^2 donor; 100 m^2 border	19.8 (178/9)[1]	Sandhill Horticulture Farm[2]	Sand
		Botany/Plant Pathology Farm[2]	Bot-1
1 m^2 donor; no border	0.0 (0/9)	Rogers Preserve[3]	Bot-2
		Palmerly Farm[2]	Palm

[1]Number of plants in the border/number of plants in donor plot
[2]Michigan State University Farms, Ingham County, MI
[3]Michigan State University Farms, Jackson County, MI

were controlled by hand until the canopy closed. In the absence of adequete rain, plots were irrigated at a rate of approximately 1.3cm of water per week. In 1993, plants were set directly into the ground. In 1994, plants were planted through plastic mulch laid out in the rows. The mulch was used to decrease moisture evaporation from the soil and raise the root zone soil temperature. When the female flowers were beginning to open, (August 11 in 1993; July 26 in 1994), one bee hive containing approximately 35,000 honeybees, (*Apis mellifera*) was placed at the edge of the donor plants in each plot (Fig. 1). The hives were removed just prior to fruit harvest.

Fruits were harvested on September 28 and 29 in 1993. In 1994, fruits were harvested as they ripened beginning on September 22 and extending through October 12. Fruits were stored in plastic bags at 0-5°C until the seeds were extracted. Following extraction, seeds were air dried for a minimum of one day and then stored in paper envelopes at 10°C and 25% relative humidity.

To evaluate long distance gene movement, seeds were bulked by the satellite from which they originated. In order to compare the movement of native and transgenes, the plot borders were divided into $1m^2$ subplots. Fruits were harvested and sorted according to the subplot from which they originated.

Seeds were germinated and scored in a greenhouse at Michigan State University, East Lansing, MI beginning in June of 1994 and continuing thru October of 1995. The seeds were planted 50 to a tray in 56 cm x 28 cm plastic seedling trays filled with a 1:1 mix of sphagnum peat perlite mix (Baccto Professional Planting Mix, Michigan Peat Co., Houston, TX) and sterilized sandy loam. From mid-November through mid-April, the seedlings were grown under artificial light ranging in intensity

from 258 $\mu mol \cdot s^{-1} \cdot m^2$ photosynthetically active radiation (PAR) on an overcast day to 530 $\mu mol \cdot s^{-1} \cdot m^2$ PAR on a sunny day. When necessary to maintain the plants longer for further analyses, seedlings were transplanted to six inch clay pots.

Progeny Screening Progeny from the recipient plants were screened for native and transgene movement. Native gene movement was scored as the percentage of green cotyledon seedlings arising from non-transgenic virescent recipient parents. If the cotyledon score was ambiguous, a second morphological trait, flower color, was scored.

Transgene movement was detected by screening all seedlings for NPT II protein using a double-antibody sandwich enzyme linked immunosorbant assay (ELISA). The NPT II assay kit was purchased from 5 Prime - 3 Prime Inc. (Boulder CO). Assays were performed according to the manufacturers specifications. Seedlings were sampled at the first or second true leaf stage. Leaf samples were taken by punching discs out of newly expanded leaves with a paper punch. Discs were placed in Corning 96 well disposable culture plates (Corning Glass Works, Corning, NY). The plates were placed in zip-lock bags and frozen at -80°C until the ELISA tests were completed. Replicate samples of each leaf were collected at each sampling.

The morphological and ELISA scores were compared for each individual seedling. In the event of an apparent discrepancy between the two scores (green cotyledon, NPT II ELISA$^-$ or vir, NPT II ELISA$^+$) the following tests were performed as necessary: (1) ELISA's were rerun with both the replicate and fresh leaf samples, (2) the second morphological trait was scored, and/or (3) genomic DNA was analyzed for the presence of the NPT II gene using (PCR) primers specific to the NPT II gene.

DNA Extraction and PCR Analysis Genomic DNA was extracted from plant leaf tissue using the miniprep procedure described by Dellaporta et al. (1985) with the following modifications: 1) Quantities were reduced to be appropriate for approximately 0.5 grams of newly expanded leaf tissue and 2) Initial extraction was performed with a mechanical pasta roller (Atlas pasta machine, Vitantonio Manufacturing Co., Italy). The leaves along with 850 microliters of DNA extraction buffer were placed in a two ounce plastic, puncture proof bag (Whirl-Paks, Baxter Diagnostics Inc., McGaw Park, IL) and run between the rollers set at the narrowest setting. Immediately after crushing, 750 microliters of the crude extract was pipetted from the bag and placed in an eppendorf tube on ice to which was added 50 microliters of 20% SDS. The tubes were placed in a 65°C water bath for 10 minutes after which 250 microliters of 5M potassium acetate was added to each tube. The tubes were placed on ice for five minutes and then spun in a microfuge at 13,750 rpms for 10 minutes. Eight hundred microliters of the supernatant was pipetted into a new eppendorf tube to which 560 microliters of cold isopropanol was added. Tubes were placed in a -80°C freezer for five minutes and then were spun in a microfuge for 10 minutes at 13,750 rpm. Isopropanol was poured off the samples and the resulting pellet was allowed to dry in the eppendorf tube. After drying, the samples were resuspended in 50 microliters of 1X TE, to which was added one-tenth volume of 3M sodium acetate, pH 5.2. To further purify the samples, they were digested with RNAse and extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The samples were then precipitated with two volumes of cold ethanol followed by a rinse with 70% cold ethanol. Samples were

dried and resuspended in double distilled water. Resultant DNA samples generally ranged in amounts from 4 to 20 ng/microliter.

To verify the presence of the NPT II gene, genomic DNA samples were used as a template for PCR. Reactions were done according to the manufacturers specifications (Perkin Elmer, Norwalk, CT). Each reaction contained approximately 100ng of template DNA and two 18 base pair primers specific to an internal 700 base pair region of the neomycin phosphotransferase II (NPT II) gene (Beck et al., 1982). Thermocycler conditions were 94°C melt for 1 minute, followed by 1 minute at 62°C for primer annealing, followed by a 2 minute extension at 72°C for 2 minutes. This cycle was repeated 45 times, followed by a 5 minute extension at 72°C. Each set of DNA extractions and PCR reactions included positive (NPT ELISA$^+$ plants) and negative (virescent NPT II ELISA$^-$ plants) controls. In several instances DNA from PCR$^+$ and/or PCR$^-$ plants was reextracted and the samples were retested to verify reproducability of the results.

Data Analysis Native gene movement was calculated as the percentage of green cotyledon or yellow flowered seedlings arising from the progeny of yellow cotyledon, white flowered parents. Similarly, transgene movement was scored as the number of NPT II positive seedlings found among the total seedlings from non-transgenic parents. To evaluate the influence of border rows on long distance gene movement, we compared levels of gene movement to the satellite plots for the borderless and bordered plots using a one-tailed t-test for samples with unequal variance; each of the individual satellites was used as a replicate.

Results and Discussion

Long distance gene movement from the bordered plots to the satellites was identical for both the native and transgene (Table 3). In each case where there was a non-virescent seedling, there was detectable NPT II protein; for each virescent seedling, no NPT II protein was detected. For both the morphological marker gene and the transgene there was significantly less long distance gene movement to the satellite plots from the bordered plots (0.0% - 0.11%) than from the borderless plots (0.27% - 2.16%)(Table 3). These results are in good agreement with previous studies on cucumber where borders were found to reduce but not eliminate gene flow from small plots (Hokanson et al., 1996).

Short distance movement of both the native and transgene within the plot borders assumed a leptokurtic distribution (Fig. 2). Movement of the transgene mirrored the pattern for the native gene. As was the case for long distance movement to the satellite plots, movement of the transgene was never detected without concommittant movement of the native gene, i.e. there were no virescent seedlings expressing the NPT II gene (Table 4).

There were, however, 39 green seedlings arising from a few subplots at the Sandhill farm that did not express the NPT II protein as measured by ELISA (Fig. 2,3; Table 4). Possibile explanations for the presence of the NPT II ELISA$^-$ green seedlings are: 1) The gene was present, but not detectable by ELISA, i.e. there was transgene inactivation, or 2) The gene was absent due to one of the following reasons: a) There was a donor plant heterozygous for the NPT II gene, b) A non-transgenic green plant could have been accidently planted in the Sandhill border plot, or c) The

Table 3. Long distance movement for the native and transgene to the satellite plots for the four sites in 1994.

			% Gene Movement[1]		
Treatment	Site	Number Screened	Native	Mean	Transgene
100 m^2 border	Sand	1106	0.0%	0.05*	0.0%
	Bot-1	901	0.11%		0.11%
No border	Bot-2	743	0.27%	1.46	0.27%
	Palm	832	2.16%		2.16%

*$p<0.05$, one tailed t-test for samples with unequal variance, df=12

[1]Scored as the percent green (native) or NPT II positive (transgene) amongst all seedlings screened.

Figure 2. Percent gene movement for the native (Vir), ○ , and the transgene (NPT II), ▲, plotted as a function of distance in meters from the donor plot center. Gene movement is calculated as the percent Vir (native) and percent NPT II (trans) postive among all seedlings scored.

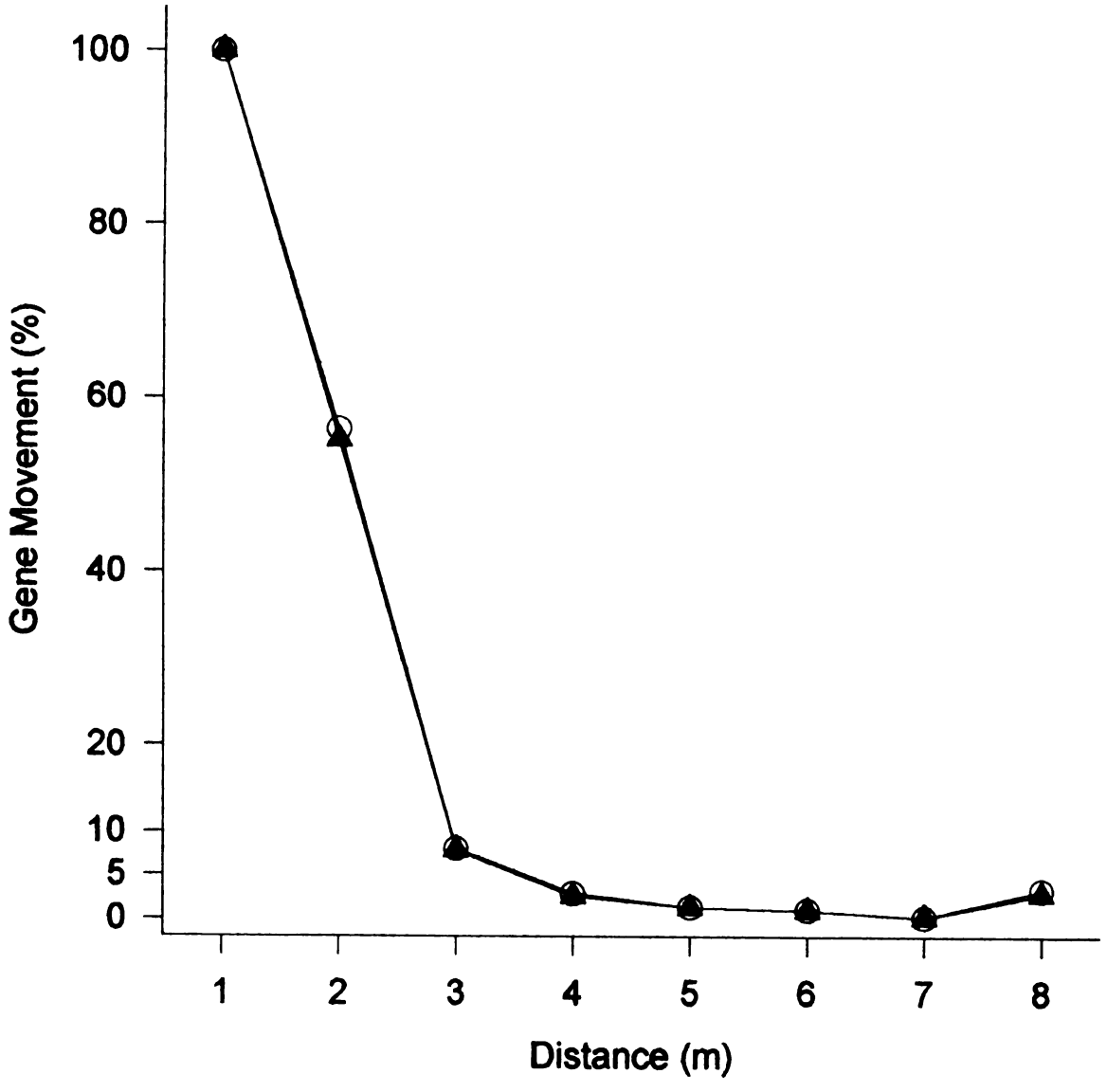

Figure 2.

Table 4. Phenotypic combinations for progeny of recipient, non-transgenic plants. Presence of NPT II was analyzed by ELISA.

Phenotype		
Native gene	Transgene	# of individuals
Vir	NPT II ELISA$^-$ (recipient phenotype)	3,860
Vir	NPT II ELISA$^+$	0
green	NPT II ELISA$^-$	39[1]
green	NPT II ELISA$^+$ (donor phenotype)	586

[1]All occurred in the Sandhill plot. See Figure 3.

Figure 3. Plot map depicting the Sandhill border subplots (numbered 1-132), and the location of the fruits which produced the 39 NPT II ELISA$^-$ green seedlings (shaded subplots). The six striped subplots in the center depict the location of the donor plants. Numbers in the shaded subplots represent; (Top), total number of green NPT II ELISA$^-$ seedlings originating from the subplot, (Bottom), total number of NPT II ELISA$^-$ seedlings found to contain an NPT II gene with PCR.

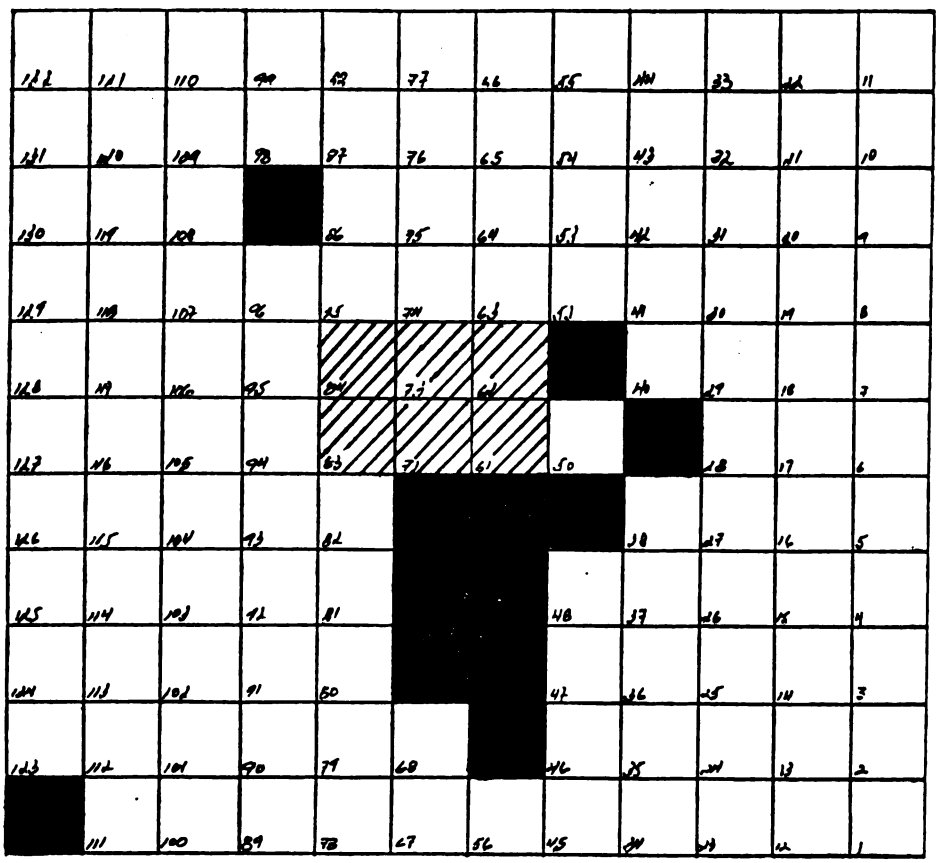

Figure 3.

native marker gene was moving in the absence of the transgene and violating normal rules of transmission genetics.

In order to determine whether transgene inactivation was responsible for the occurrence of the apparently NPT II ELISA$^-$ seedlings, genomic DNA was extracted and used as a template for amplification of the NPT II gene via PCR. For 27 of the 39 NPT II$^-$ plants PCR analysis revealed the presence of a band corresponding to the predicted 700 base pair NPT II fragment (Fig. 4). The presence of the NPT II gene fragment in the absence of detectable NPT II protein indicates transgene inactivation as the explanation for the apparent discrepancy between the native and engineered gene. These results suggest that transgene inactivation occurred in at least one donor plant. We have also observed other transgenic melon lines for which NPT II gene expression has been silenced (Grumet, unpublished).

To further verify that the 12 remaining NPT II ELISA$^-$ plants did not contain the NPT II gene, four of the 39 heterozygous NPT II$^-$ plants were self pollinated in the greenhouse. The progeny of three segregated as predicted for the native gene, three green; one vir (154:57; $\chi^2 = 0.456$, df =1). As was the case for the parents, none of the resultant F_2 progeny (n = 211) expressed detectable levels of NPT II protein by ELISA (data not shown), nor could the NPT II gene be detected with PCR (n=20). The segregation of selfed progeny from the fourth plant indicated that a heterozygous green, (Vv), non-transgenic mother may have been the donor of the 12 NPT II$^-$ plants. The progeny of this plant were all green. This outcome could not be explained by a donor plant that was heterozygous for the NPT II gene (1/4 of the progeny would be expected to be vir). Importantly, this outcome also could not be explained by

Figure 4. PCR products resulting from a reaction run with the two 18 base pair primers flanking a 700 base pair region internal to the NPT II gene, and genomic DNA from: Lanes 2-3, NPT II ELISA$^+$ green seedlings; Lane 4, NPT II ELISA$^-$ virescent seedling; and Lanes 5-10, NPT II ELISA$^-$ green seedlings. Band in lanes 2-3 and 5-10 corresponds to approximately 700 base pairs based on comparison with the Hind III digested λ DNA size standard, Lane 1.

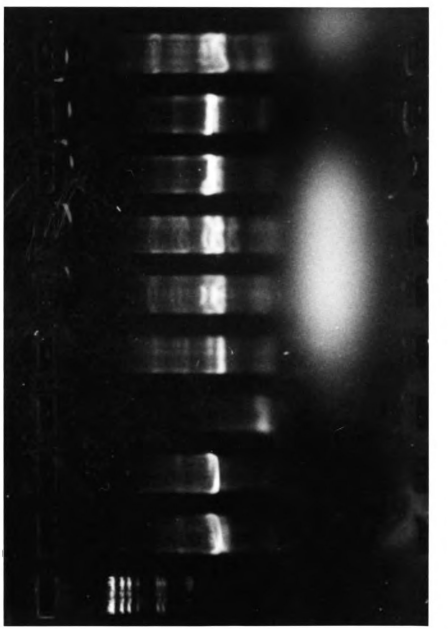

Figure 4.

differential movement of native and engineered genes (again 1/4 of the progeny would be expected to be vir). This outcome, (all green progeny) only could have occurred if this fourth plant was homozygous for the non-virescent (VV) allele. For that to happen, it must be the offspring of a heterozygous green, (Vv), non-transgenic mother that was an accidental contaminant in the border plot. We have noted on occasion a rare green cotyledon seedling in the virescent seed lots which are typically rouged. Unfortunately it appears one escaped rouging to be planted in the plot border. The various progeny we noted then would be the results of fertilizations from a mixture of pollen including; self-pollen from the mother (which created VV, Vv and vv NPT II negative progeny), pollen from the homozygous green transgenic donors (creating VV and Vv NPT II positive progeny) and pollen from homozygous yellow, non-transgenic border plants (creating Vv and vv NPT II negative progeny).

The occurrence of the 27 plants in this study which were NPT II ELISA$^-$, but were later found to be NPT II positive by PCR has implications for any study designed to measure transgene movement. Studies which only screen for the presence of a selectable marker (gene product), such as herbicide or antibiotic resistance, run the risk of underestimating the actual levels of transgene movement due to transgene inactivation phenomenon. In order to avoid these underestimates, the most thorough way to analyze progeny in such studies would be to screen for the presence of the actual transgene using PCR or Southern analysis. However, these progeny screens would be enormously expensive and time consuming. The presence of a second marker in our studies, (green cotyledons), allowed us to detect outcrossing in the absence of NPT II expression. The use of such a dominant marker gene to identify

potential outcrossers as a primary screen, coupled with a secondary screen for actual transgene presence in a more limited population presents a tractable way around dollar and time constraints in such studies.

In conclusion, our data support the assumption that native and transgenes have the same pollen-mediated gene dispersal patterns. This confirmation allows for the confident use of a rich body of information on non-transgenic plants in making predictions about patterns of gene dispersal via pollen in transgenic plants. Although gene flow information is an important component relative to the establishment and spread of transgenes in natural ecosystems, other factors must also be considered. Specific features of the particular transgene (i.e. does it confer a selective advantage) will strongly influence performance of the progeny, this in turn will effect the rate and extent of establishment and spread (Williamson, 1994; Regal, 1994; Gliddon, 1994; Linder and Schmitt, 1994; Gabriel, 1993).

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CONCLUSIONS

CONCLUSIONS

The objectives of this project were to: 1) Investigate the efficacy of border rows as a strategy to limit the pollen-mediated escape of transgenes from crop plantings and 2) Determine whether native and transgenes have the same pollen-mediated dispersal patterns.

Border rows have been incorporated into many of the field tests of genetically engineered crops to satisfy requirements for restricting the escape of engineered genes. Although a limited number of studies have investigated the movement of transgenes into a contiguous border of non-transgenic cogeners, no systematic study of the influence of border rows on pollen-mediated long distance gene movement to isolated patches of co-geners has been reported. Research along these lines is important because such an experimental design closely approximates the situation where patches of crop wild relatives surround commercial crop plantings.

Our results demonstrate that border rows can have a significant influence on long distance gene movement to discontiguous satellite plots. As the trap plant to donor plant ratio increased from 0 - 131, there was a significant decrease in long distance gene movement. In the plots with a ratio of 131 we saw no long distance gene movement into the satellites.

Despite the significant treatment effect observed in these experiments, the

border row strategy has several inherent weaknesses. The principle problem is economic in nature. Even our smallest trap/donor ratio which allowed on average 0.61% outcrossing to the satellite plots, would necessitate the planting of 11.6 acres of border plants for every acre of transgenics. This does not appear to be an economically viable strategy for a grower. From an environmental perspective, some might find the less than perfect protection afforded by the strategy objectionable.

The other significant issue raised by this study concerns environmental variation. Year to year and site to site variation in long distance gene movement suggests that broad predictions regarding the influence of border rows as a containment strategy can not be made. The 38% outcrossing rate observed in one of the satellites is an example of a "low frequency, high magnitude" type event some environmentalists fear could result from the unregulated commercialization of genetically engineered crops.

Results from our experiments compel us to conclude that regardless of the border row strategy employed, some level of escape of engineered genes from agronomic-scale plantings appears inevitable.

Much of the response to issues regarding the potential risks of genetically engineered crops has been based on a long standing body of information generated from research with non-transgenic organisms. Application of such information to risk assessment for transgenic plant issues necessitates the assumption that there is no inherent difference in pollen-mediated dispersal patterns for native and transgenes. While this assumption is probably valid, it remains untested. To our knowledge, no reports exist where native and transgene dispersal patterns are compared.

Long distance dispersal patterns of the native and transgene in this study were identical. At no time did we see movement of the transgene without a concomittant movement of the native gene or vice-versa. Regarding short distance movement into the plot borders, the dispersal patterns for the two genes were nearly identical. The slight difference observed for short distance dispersal, (0.30%), was accounted for by the unfortunate occurrence of a contaminant plant in one of the plots. Aside from this, there was no unaccountable difference in dispersal patterns between the two genes. These results suggest that the application of information generated from research on non-transgenic organsims will be legitimate and useful.

Two important cautionary issues must be raised at this point. The first is the phenomenon of transgene inactivation. Transgene inactivation is basically the lack of expression of the transgene itself and/or endogenous genes, apparently resulting from the insertion of DNA into the genome via the transformation process. The phenomenon has resulted in some unpredicted phenotypes which in some cases appeared to be the result of abberant transmission patterns. Discovery of 27 NPT II $ELISA^{-}$ PCR^{+} seedlings originating from the Sandhill plot in these experiments (presumably a result of transgene inactivation) raises important implications for any similar experiments evaluating the risk of pollen-mediated escape of engineered genes. Studies designed to look only for presence of a transgene product such as herbicide or antibiotic resistance, as a measure of transgene movement, run the risk of underestimating the actual amount of gene movement due to the transgene inactivation phenomenon . A true measure of transgene movement can only be gained through screening for presence of the actual transgene. In the case of our experiments, the

difference in the two measures would have been nearly one percent.

The second caution does not arise directly as a result of this research, nor is it new. The fact that the dispersal patterns for native and transgenes in this study were the same does not suggest that the transgenes will not have a unique impact in the natural environment. It is important that the adaptive value of each gene be evaluated individually. Each gene has the potential to impart on its new host a particular fitness value which could make that transgenic plant more or less competitive relative to its neighbors. In fact, with the ability to move genes between genera, families and even kingdoms, scientists have the ability to impart on plants fitness traits and thus selective potentials never seen before in the plant community! Persistance and spread of transgenes in the environment are not parameters concerning risk assessment about which we can necessarily make assumptions. Such decisions will to a large degree be made on a gene/crop by gene/crop basis.

Given the high likelihood of some level of escape of transgenes from commercial scale plantings, the highest priority for future risk assessment research should be in the area of establishment and spread of transgenes in the environment. Specifically, can the risk of escape of transgenes into the environment be mitigated at this level? The focus of such research should be on the influence of the selective value imparted by a particular transgene on the likelihood of its persisting and spreading in the environment. The advent of biotechnology presents an excellent opportunity to "build" plants with a quantifiable fitness value in a given environment, i.e. herbicide resistance under certain herbicide spray regimes. Experiments could be designed to test the persistance of and rate of spread of transgenes under selection

conditions which reduced the fitness of non-transgenic controls by 25, 50 or 75%. Such direct, quantitative selection experiments would provide valuable information to both the risk assessment and evolutionary biology communities.

With such background information in hand, predictions regarding the potential risks posed by specific gene/crop combinations might be made. At the least, such information would allow more informed decisions to be made regarding which gene/crop combinations merit the most rigorous oversight and investigation.

Such prioritising is critical as agricultural biotechnology firms seek to begin gaining some profit from their investments. Strict regulations over all products of biotechnology will result in one of two eventualities. Lack of profits could prompt biotech firms to discontinue biotech pursuits. Alternatively, strict across the board regulation could result in a move to end all regulation of biotech products. Neither scenario is acceptable. Although the present day products of agricultural biotechnology are not producing "gardens in the desert", at some time in the future they will in all likelihood do that and more. To acheive that end the industry needs to be allowed to prosper. However, we can not allow progress at any cost. Just as certainly as there are products of biotechnology which are overregulated today, there are products in the pipeline which will merit strict oversight.

With all said and done, risk assessment of genetically engineered crops will to a large extent be relegated to a case by case basis. Certain crop/gene combinations may well be deemed unallowable, while others will necessitate as little oversight as present day conventionally bred crop varieties.

APPENDICES

Appendix 1. Reduction in reproductive capacity (flower and fruit production) of non-transgenic cucumber variety 'Wisconsin SMR-18' due to spray treatments with "Pinnacle" herbicide, (Dupont DPX-M6316), 1992 experiments.[1]

	♂ Flowers/plant[2]		♀ Flowers/plant		Fruit/Plant[3]	
Treatment[4]	50 DAP[5]	58 DAP	50 DAP	58 DAP	58 DAP	92 DAP
0.0 g	3.4 (±0.14)	14.1 (±0.88)	0.3 (±0.24)	4.1 (±0.44)	1.0 (±0.47)	2.8 (±0.43)
1.1 g/hectare	2.7 (±0.90)	14.3 (±2.7)	0.3 (±0.24)	3.5 (±0.34)	1.5 (±0.48)	3.0 (±0.28)
2.2 g/hectare	3.9 (±0.51)	13.5 (±1.9)	0.5 (±0.17)	4.3 (±0.86)	1.3 (±0.47)	2.4 (±0.29)
4.4 g/hectare	2.2 (±0.36)	12.1 (±0.90)	0.3 (±0.19)	3.6 (±0.91)	1.5 (±0.48)	2.4 (±0.64)
8.8 g/hectare	1.1 (±0.49)	7.1 (±0.98)	0.2 (±0.10)	2.4 (±0.40)	0.5 (± 0.22)	2.5 (± 0.41)

[1]RCBD, four reps, 5 treatments per rep, 3 plants per treatment. Plants were spaced .5 m within the row, 71 cm between rows. Herbicide treatments were applied at the four leaf stage.

[2]Flower numbers are per plant averages per treatment averaged over four reps (± standard error).

[3]Fruit numbers are per plant averaged over four reps (± standard errors). All fruits > 2 inches in length were counted.

[4]Treatment amounts are grams of active ingredient (Pinnacle Df 25 herbicide) per hectare.

[5]DAP-days after planting

Appendix 2. Reduction in reproductive capacity (flower and fruit production) of non-transgenic cucumber variety 'Wisconsin SMR-18' due to spray treatments with "Pinnacle" herbicide, (Dupont DPX-M6316), 1993 experiments.[1]

	♂ Flowers/plant[2]		♀ Flowers/plant		Fruit/Plant[3]	
Treatment[4]	39 DAP[5]	46 DAP	39 DAP	46 DAP	46 DAP	85 DAP
0.0 g	2.3 (±0.56)	9.2 (±1.2)	1.0 (±0.41)	2.1 (±0.16)	1.8 (±0.52)	2.8 (±1.4)
4.4 g/hectare	0.1 (±0.10)	1.0 (±0.27)	0.4 (±0.09)	1.4 (±0.50)	0.5 (±0.10)	32.4 (±0.36)
8.8 g/hectare	0.2 (±0.12)	0.5 (±0.36)	0.0 (±0.0)	0.8 (±0.50)	0.1 (±0.10)	2.0 (±0.0)
17.6 g/hectare	0.1 (±0.10)	0.4 (±0.14)	0.1 (±0.13)	0.5 (±0.21)	0.0 (±0.0)	0.9 (±0.43)
26.4 g/hectare	0.1 (±0.10)	0.1 (±0.10)	0.0 (±0.0)	0.4 (±0.21)	0.0 (± 0.0)	1.5 (± 0.57)

[1]RCBD, four reps, 5 treatments per rep, 3 plants per treatment. Plants were spaced .5 m within the row, 71 cm between rows. Herbicide treatments were applied at the four leaf stage.

[2]Flower numbers are per plant averages per treatment averaged over four reps (± standard error).

[3]Fruit numbers are per plant averaged over four reps (± standard errors). Fruit counts at 46 DAP were all fruit > 2 inches in length. Fruit count at 85 DAP were mature fruit only.

[4]Treatment amounts are grams of active ingredient (Pinnacle Df 25 herbicide) per hectare.

[5]DAP-days after planting