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Risk of alfalfa transgene dissemination and scale-dependent effects

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Abstract Pollen can function as a vehicle to disseminate introduced, genetically engineered genes throughout a plant population or into a related species. The measurement of the risk of inadvertent dispersal of transgenes must include the assessment of accidental dispersion of pollen. Factors to be considered include the rate of pollen spread, the maximal dispersion distance of pollen, and the spatial dynamics of pollen movement within seed production fields; none of which are known for alfalfa (*Medicago sativa* L.), an insect-pollinated crop species. Using a rare, naturally occurring molecular marker, alfalfa pollen movement was tracked from seed and hay production fields. Results indicated that leafcutter bees (*Megachile* spp.) used in commercial seed production show a directional, non-random bias when pollinating within fields, primarily resulting in the movement of pollen directly towards and away from the bee domicile. Within-field pollen movement was detected only over distances of 4 m or less. Dispersal of pollen from alfalfa hay and seed production fields occurs at distances up to 1000 m. By examining widely dispersed, individual escaped alfalfa plants and their progeny using RAPD markers, gene movement among escaped alfalfa plants has been confirmed for distances up to 230 m. The outcrossing frequency for large fields was nearly 10-times greater than that of research-sized plots. A *minimum* isolation distance of 1557 m may be required to prevent gene flow in alfalfa. Data suggest that complete containment of transgenes within alfalfa seed or hay production fields would be highly unlikely using current production practices.

Key words Escaped plants · Gene flow · Pollen dissemination · *Medicago sativa* L. · *Megachile* spp.

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Introduction

The use of genetic engineering to improve crop plant performance has become a reality that will increase dramatically in frequency as time progresses. As of late 1998, there have been 4666 approved releases of transgenic organisms into the environment in the United States (online records at http://www.nbiap.vt.edu), and undoubtedly many more throughout the world. More than 50 species of flowering plants have been genetically engineered and released into the environment, and many more species are current targets of genetic engineering. The kinds of genes currently used in the genetic engineering of crop plants can be broadly classified as those conferring resistance to chemicals, abiotic stress, or pests; altering product quality; or altering agronomic traits. Engineered genes may come from microbes, plants, or animals and alter the phenotype of crop plants. The movement of genes across species boundaries presents many opportunities for both expected and unexpected risks. For example, in a study of allergenicity, when modified soybeans (*Glycine max*) containing a gene from Brazil nut (*Bertholletia excelsa*) were tested on people known to be allergic to the nuts, they showed an allergic reaction to the modified soybeans (Nordlee et al. 1996).

In addition to food safety, other serious concerns involve ecological risks, such as new or increased pest resistance to insecticides and herbicides due to hybridization or excessive selection pressure, changes in the ecological competency of crops, and the possible loss of genetic diversity in areas of crop origin. For example, transgenic sorghum (*Sorghum bicolor*) containing a her-

bicide resistance gene, could hybridize with Johnsongrass (*Sorghum halepense*), a noxious weed in regions where hybrid sorghum seeds are produced. Successful *S. halepense*×*S. bicolor* crosses have been documented to occur up to 100 m from cultivated sorghum (Arriola et al. 1995; Arriola and Ellstrand 1996). Clearly, widespread cultivation of transgenic, herbicide-resistant sorghum would lead to herbicide-resistant Johnsongrass in the surrounding area, quickly limiting the effectiveness of the transgenic sorghum and creating a more noxious weed.

If transgenic plants carry a gene that confers resistance to a pathogen or insect pest, the release of that gene into the environment may apply selection pressure on the pest population, resulting in an increase in resistant pests and a loss of effectiveness of the resistance gene. For example, there currently are licensed cultivars of maize, cotton, and potato for commercial production, which contain the gene that codes for an endotoxin from *Bacillus thuringiensis* (Bt). In a study to determine the frequency of naturally occurring resistance to the Bt toxin in *Heliothis virescens*, perhaps the main target pest for which Bt cotton was developed, it was found that resistance alleles occurred with a frequency of 1.5 per 1000 (Gould et al. 1997). This surprisingly high frequency does not bode well for the longevity of field effectiveness of the Bt gene in the transformed plants.

The unintended dispersal of transgenes into surrounding populations should be addressed from at least three perspectives; the ecological impact of escaped transgenic plants, the ecological impact of transgenic-wild type hybrids, and the impact of "escaped" transgenics on pest populations. Considering plant populations, the first concern is that seeds of the transgenic crop may be dispersed either through natural processes, such as animals feeding on the mature crop and disseminating the seeds, or by human intervention such as crop material falling off farm machinery during transport. For example, after the harvest of canola seeds and subsequent transportation to processing centers, some canola seed will be distributed along roadsides. If the crop is one of the currently available transgenic herbicide-resistant varieties, the plants that grow along the roadsides will also be herbicide resistant. The ecological impact of this fact is difficult to predict. Is there an ecological effect to suddenly having a large number of herbicide-resistant canola plants growing along the roadsides of canola-producing areas? Some existing plant populations may be displaced, but any long-term effects of such an event will vary with the introduced species. Two major factors determining the level of impact of escaped transgenics are whether the introduced species can propagate in the surrounding area and whether the species is cross compatible with weeds in the area.

Baranger et al. (1995) have documented the transfer of transgenic herbicide resistance across *genera*! In that case, transgenic rapeseed (*Brassica napus*) hybridized with weedy *Raphanus* species and the hybrid progeny expressed herbicide resistance. The hybrids were able to form progeny with *Raphanus*, indicating that second- and higher-generation dispersal was also possible. Eventually, herbicide-resistant *Raphanus* could find its way into rapeseed fields, and the intended herbicide would be worthless. The level of risk for this kind of dispersal depends in large part on the dispersal patterns of the pollen of the species in question. Measurements of this dispersal are vital for developing containment strategies.

In general, pollen dispersal studies have taken two forms, either source populations were planted within larger fields and pollen movement away from the source areas measured; or pollen "sinks", small groups of plants widely separated from all known plants of the same species, were established and the seeds produced on them were monitored for non-self events. Usually, the studies measuring pollen movement out of a source planting into a larger surrounding field indicated that pollen moves over very short distances (typically 5 to 20 m) (e.g. Conner and Dale 1996; Llewellyn and Fitt 1996). In "pollen sink" studies, the opposite was true; pollen movement of 1000 m has routinely been reported (e.g., Arias and Rieseberg 1994). With wind-pollinated plants, such as evergreen trees, it is likely that pollen could be vectored for great distances, covering many kilometers. A study of sunflower (*Helianthus annuus* L.) pollen movement demonstrated that pollen was carried at least 1000 m (Arias and Rieseberg 1994). It is not known whether wind or insects, or both, carried the pollen that distance.

The size of the area planted to a transgenic crop, or the pollen "load", undoubtedly affects the dynamics of pollen dispersal. For example, it seems unlikely that pollen will travel the same distance and direction from a 1-m^2 plot as from a 100-acre commercial field. Ellstrand (1988) suggested the use of trap plots for monitoring pollen movement away from pollen sources, and demonstrated that movement over large distances is common under some conditions.

Alfalfa (*Medicago sativa* L.) is amenable to genetic engineering; currently, 19 permits have been issued to release transgenic alfalfa into the environment in the USA. Because alfalfa is easily genetically transformed and produces large amounts of protein, it is often considered as the ideal plant to use in the large-scale production of transgene products. Alfalfa is an outcrossing crop plant with cleistogamous flowers, relying entirely on insects, primarily bees, to effect pollination. The alfalfa floral system is unique among cross-pollinated field crops in that the explosive tripping mechanism (Barnes et al. 1972) virtually guarantees that only the first pollinator visit will result in seed formation. Therefore, the pollen-dispersal dynamics of alfalfa will most likely be considerably different from that of other systems, and may serve as a baseline for the study of other plant systems subject to similar pollination mechansims.

Little is known of the distances and patterns of pollen dispersal by insects from alfalfa. There are no known weedy relatives with which alfalfa will inter-mate in the US; however, noncultivated populations of alfalfa grow-

ing on roadsides are common. Hence, one of the main concerns with transgene dispersal from alfalfa fields is the establishment of feral populations with unintended expression of a transgene. An additional concern is that seed-production fields may be subject to contamination from neighbouring fields, compromising varietal purity and resulting in a portion of the seed produced carrying the transgene-encoded trait.

To reasonably assess the risks associated with alfalfa transgene dispersal, and to develop workable containment strategies, it is necessary to collect data on the dispersal of alfalfa pollen from seed and hay production fields. The objectives for this study were: (1) to determine the spatial dynamics of pollen movement within alfalfa seed production fields, (2) to assess the movement of pollen away from seed and hay production fields, (3) to investigate the movement of pollen between escaped alfalfa plants, and (4) to address the effect of field size on pollen movement.

Materials and methods

Spatial dynamics of pollen movement within seed production fields

Genetic marker and detection

Several PCR primer pairs were designed to flank intron sequences in the published alfalfa glutamine synthetase (GS) gene (Tischer et al. 1986). Primer pairs were tested on more than 300 unrelated alfalfa genotypes to identify naturally occurring polymorphic DNA sequences using standard PCR techniques (Griffin and Griffin 1994). A single plant, Varia 74 from "Varia" basic germplasm source PI 536533, was identified which carried a fragment of approximately 200 bp that was not present in any of the other genotypes examined. The polymorphic DNA was partially sequenced using a modified Sequenase technique (Trewick and Dearden 1994) and primers specific to the polymorphic DNA were synthesized. These primers increased the specificity of the amplification reactions and were used routinely to generate the specific polymorphism found in Varia 74. Subsequently, a second genotype with the polymorphism was identified; it was also from within the Varia germplasm (plant Varia 57). The two individuals carrying the specific polymorphism were crossed and the progeny intercrossed and screened over several generations to develop source plants homozygous for the marker band. The sequence of the marker band was compared to the published alfalfa GS gene using ClustalW (Thompson et al. 1994) and to GenBank using BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988).

Detection of the marker band in progeny plants was performed using standard PCR techniques (Griffin and Griffin 1994). Total DNA was extracted from entire seedlings using the NaOH method (Wang et al. 1993). In order to reduce the total number of plants examined and to increase the total number of pollination events examined, only one seed per pod was tested, since all seeds in a given pod most likely resulted from a single pollination event.

Field design

In order to track the distance and direction of pollen movement from source plants, a circular field-plot design was used. Five plants homozygous for the marker were planted in a 1 m-diameter plot in the center of a rectangular 600×400 m alfalfa seed production field, planted with the cultivar 'Vernal', near Prosser, Washington State, USA, in the spring of 1994. A single leafcutter (*Megachile* spp.) beehive was placed along the south edge of the field at the initiation of bloom. Standard seed-production practices were followed. Each plot was laid out to maintain the same radial plot angle and plot area (0.78 m^2). This was accomplished by decreasing plot depth as plots proceeded from the source plants. Plots radiated out from the source plants at eight regular angles coinciding with the eight cardinal points of the compass. Plots were spaced 2-m apart, beginning 2 m from the source plants and proceeding out to 10 m. The measured distance for each plot was the maximum distance of the plot from the source plants. Radial plot width was 11.25 degrees. Each plot was hand-harvested and all pods in a plot were collected.

Data analysis

Transfer of the genetic marker from source plants to surrounding plants via pollen was measured using per-plot frequencies of progeny containing the polymorphic DNA marker. The mean direction of movement (mean vector) relative to the beehive, and the angular variance and deviation relative to the beehive (Batschelet 1981), were also measured. Statistical evidence for non-random direction of pollen movement was tested using the Rayleigh test (Batschelet 1981). All plots within a field were pooled for a given direction to determine the mean vector, the angular variance and deviation, and for the Rayleigh test. All data were corrected for grouping according to Batschelet (1981) and, where appropriate, axial bimodal data angles were doubled (Batschelet 1981).

Pollen movement from seed and hay production fields

Genetic markers and detection

The naturally occurring polymorphic GS marker described above was used as a source-plant marker. The RAPD primer OPH-15 (AATGGCGCAG) from Operon Technologies, Inc., was also used to generate polymorphic bands for the detection of outcrossing. A single selection from KS108 germplasm (Sorensen et al. 1985) lacking the GS marker was vegetatively propagated and used as a pollen-trap plant. Presence of the GS marker and RAPD bands in progeny plants were detected using standard PCR and RAPD methods (Griffin and Griffin 1994).

Field designs

In the spring of 1995, trap plots each with 15 plants in a 1-m-diameter area were planted along road sides 0, 20, 40, 60, 80, 100, 200, 300, 400, 500, 750, and 1000 m away from large alfalfa fields. The area containing trap plants was searched for escaped alfalfa plants on two occasions through the growing season and the few plants found were removed. Hay and seed production fields were planted at two locations. One location, Prosser, Washington, is an area of high pollinator activity with many leafcutter bees released especially for alfalfa pollination. The second location, Manhattan, Kansas, normally has no alfalfa seed production, but does have a large acreage of alfalfa hay production. The seed field at Prosser was 20 m×70 m and the hay field was approximately 120 m×200 m. The seed field at Manhattan was 20 m× 60 m and the hay field was 90 m×120 m. Hay was harvested from hay production fields five times at the Prosser location and three times at Manhattan in 1995. A 2-m^2 plot of approximately 300 source plants homozygous for the GS marker was planted at the edge of each hay and seed production field. This planting plan, and the marker systems used, allowed the testing of different-sized pollen sources in the same physical space, under the same environmental conditions, and at the same time.

Variables measured and data analysis

All seeds from trap plants were collected by hand and analyzed for the presence or absence of the GS marker described above and for

outcrossing using RAPD primer OPH-15. Marker and outcrossing frequencies were calculated for each trap-plot location. Residual analysis indicated correlations between means and variances for frequency data; therefore, all frequencies were transformed using the angular (arcsine square root) transformation prior to statistical analysis. Simple regression analysis and tests for the heterogeneity of slopes were performed using PROG REG and PROC GLM of SAS (SAS Institute, Cary, N.C.).

Pollen movement between escaped plants

Field design

Escaped alfalfa plants were located along roadsides in and near Prosser, Washington. Individual plants were also planted in urban areas at least 800 m from known alfalfa fields. The area around each individual plant was thoroughly searched for the presence of other alfalfa plants. The distance between the individual escaped plants found and the nearest alfalfa plants was measured with either an optical rangefinder (Rangematic 1200, Ranging Co., East Bloomfield, N.Y.) or with an automobile odometer. All escaped plants evaluated were located along roadsides in cultivated regions, facilitating the detection of individual plants.

Genetic marker and data analysis

RAPD markers were used to assess whether the progeny of escaped plants were the result of self- or cross-pollination. Minimum distances of gene flow were calculated for each maternal plant based on the closest distance between each maternal plant and the nearest possible paternal plant. Outcrossing frequency was calculated for each escaped plant.

Results and discussion

Marker system

A rare, naturally occurring, molecular marker was found using primers that flank introns of the alfalfa glutamine synthetase gene (Tischer et al. 1986). The GS marker is highly related to intron number six of the glutamine synthetase gene found in both alfalfa and yellow lupine (*Lupinus luteus*). Specific primers (GS M1 5´GGTGAAAACTCTTTTACACTTG3´, and GS M91 5´ACAAAAACATAGTAAATCTCTAGGG3´) were designed based on the polymorphic sequence. PCR using these primers and an annealing temperature of 54°C carried out on DNA from plants homozygous for the marker produces a single 91-b marker band. Plants not carrying the marker fragment produce a band of approximately 150–200 bp. Progeny of marker by non-marker crosses have both bands. Examination of nearly 2000 plants randomly selected from the nine basic sources of alfalfa germplasm indicated that this sequence is not present in a broad range of alfalfa genotypes (data not shown). Detection of outcrossing from plants other than the GS marker plants was performed using RAPD markers (Fig. 1). RAPD markers have been shown to segregate according to qualitative genetic theory; however, the possibility of new markers forming from crossovers in a

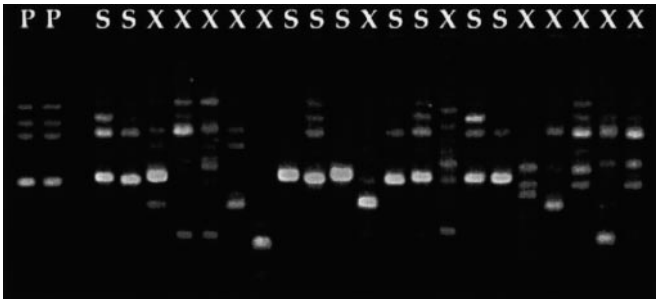

Fig. 1 Detection of outcrossing with RAPD markers. Polymorphic RAPD bands from the cloned maternal parent (*P*), used as a trap plant, and its segregating progeny which are from self (*S*) or cross (*X*)-pollination events. Selfed progeny have only maternal bands, while apparent crosses have additional bands

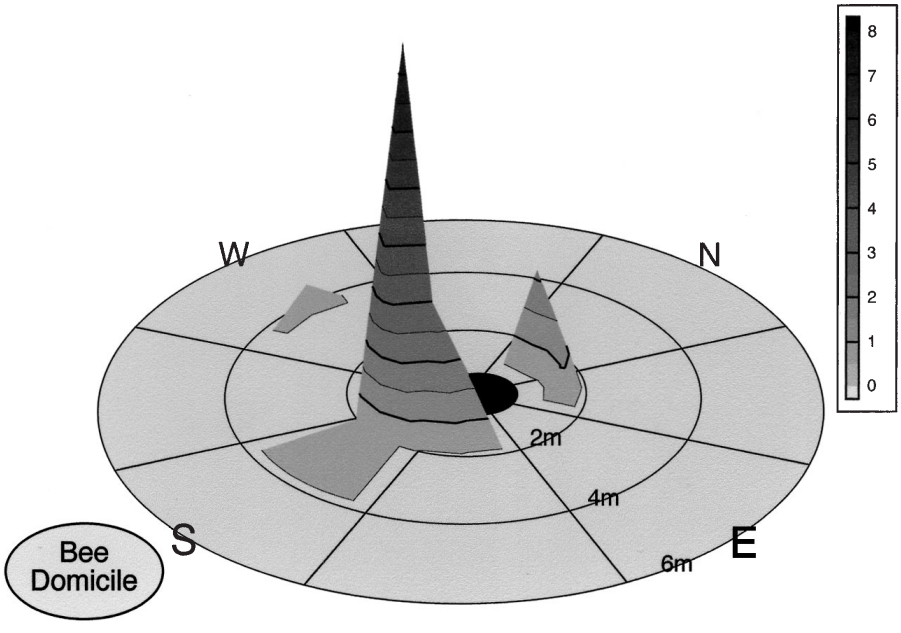

Fig. 2 Within-field marker gene movement. Five plants homozygous for a marker gene were planted in a 1 m-diameter plot in the center of a 600×400 m alfalfa seed production field in Prosser, Washington, USA. A single leafcutter (*Megachile* spp.) bee domicile was placed along the south edge of the field at the initiation of bloom. *Shading* indicates detected marker gene frequencies as a percentage of the seeds harvested from that plot

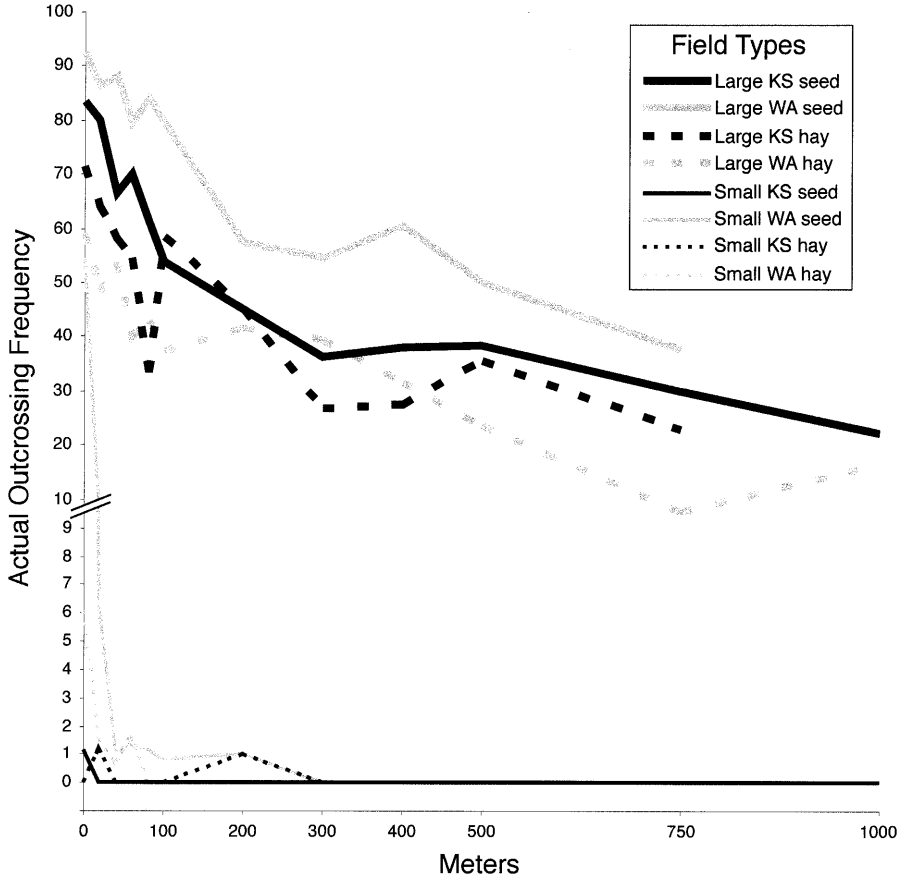

Fig. 3 Gene movement away from commercial-scale (large) seed and hay production fields and research-scale (small) seed and hay plots in Kansas and Washington State. Progeny from trap plots of cloned alfalfa were examined for a specific marker gene and for apparent non-self events using RAPDs. Outcrossing from small plots was not detected beyond 200 m (note axis scale change)

self-pollination event, though rare, may exist. RAPD markers unique to progeny and completely lacking in maternal plants were assumed to have come from a cross-pollination event.

Pollen movement within seed fields

Nearly 6000 progeny from 24 plots in a Prosser seed production field were evaluated (Fig. 2). Of those progeny, 3.06% carried the GS molecular marker. Movement of the marker within the field occurred only over very short distances. Only 0.2% of the progeny 4-m away from the marker plants carried the marker gene and no progeny 6-m away were detected with the marker. Of those progeny carrying the GS marker, 97.4% occurred no more than 2-m from the source plants. Clearly, within-field gene movement only occurs over very short distances. It is likely that pollen is being cleansed from the pollinators or is being covered with non-marker pollen after repeated visits to non-marker plants. This finding indicates that borders surrounding seed production fields may be useful in decreasing or limiting gene flow outside of a field.

Results indicate that leafcutter bees used in commercial seed production preferentially work in straight transects directly away from and towards the colony domicile when pollinating alfalfa, resulting in the movement of pollen from marker plants directly toward and away from the colony domicile (Fig. 2). Of those progeny carrying the marker, 84.6% were located directly north or south of the marker plants transecting the bee domicile. The standard deviation about the mean vector was 28.4 degrees, indicating that bees deviated little from the preferred straight transect. The majority of pollen movement (61.5%) was from the marker plants towards the domicile, indicating that bees may pollinate a field more efficiently or more vigourously as they move toward the domicile. The distribution of marked progeny around the marker source plants differed significantly ($P \leq 0.0001$) from a random distribution as measured by the Rayleigh test.

Pollen movement from alfalfa fields

Long-range dispersal of genes from alfalfa hay and seed production fields by pollen has been confirmed for distances up to 1000 m using small trap plantings of alfalfa (Fig. 3). Greater distances would most likely have been noted if trap plantings further from source plantings had been made. Outcrossing frequencies as high as 93% were detected in plots near large pollen sources. Marker genes were detected only up to 200 m away from research scale plots (Fig. 3); while RAPDs detected gene flow up to 1000 m from the larger, near commercial

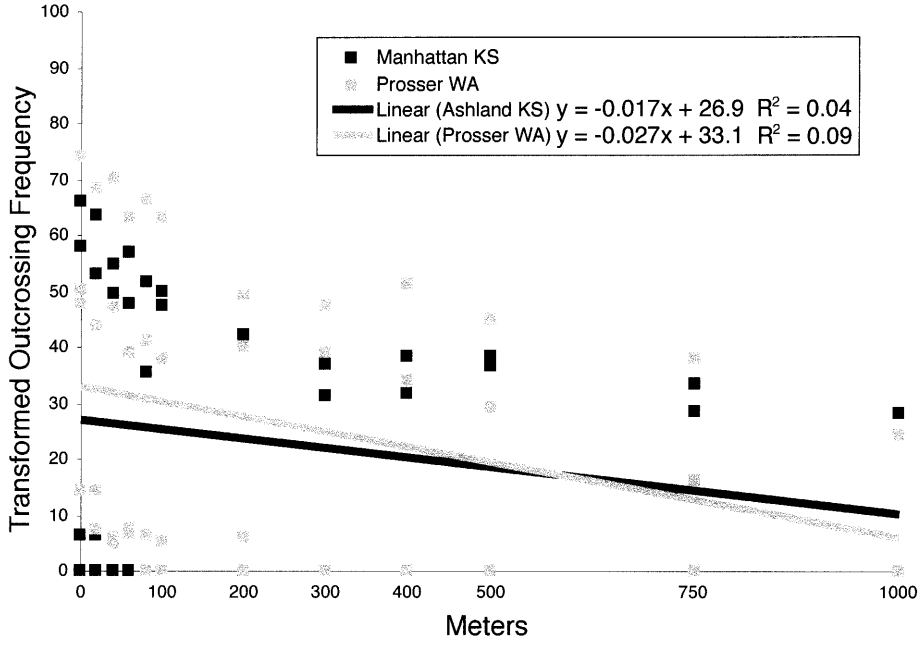

Fig. 4 Gene movement away from alfalfa fields in Kansas vs Washington State. Data pooled within locations indicate no significant differences between locations for outcrossing frequency (intercepts, P=0.4625) or for rate of decline in frequency (slopes, P=0.5715)

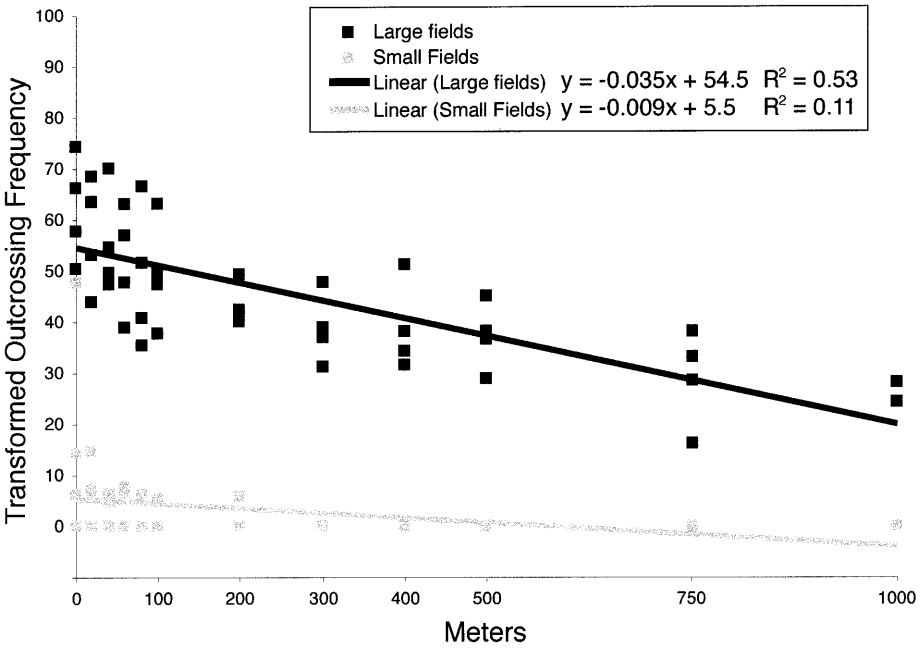

Fig. 5 Gene movement away from commercial-scale (large) fields vs research-scale (small) plots. Data pooled across locations for field size indicate significant differences between field sizes for the detection of outcrossing frequency (intercepts, P=0.0001) and for rate of decline in frequency (slopes, P=0.0001)

scale plots for both seed and hay production fields. At 1000 m, up to 22.2% of the seed resulted from outcrossing. Clearly, pollen movement beyond the current isolation distances for certified (50 m) and foundation (275 m) seed does occur.

Tests located at Prosser always had higher outcrossing frequencies than those at Manhattan (Fig. 4), but the differences were not significant (P=0.4625). The rate of decline in outcrossing frequency also was not significantly different between the two sites. The Prosser location has a high level of pollinator activity, compared with the Manhattan location, so it was surprising to find that no overall differences could be attributed to location effects.

The effects of field size on gene movement were pronounced (Fig. 5) and highly significant ($P \leq 0.0001$). Marker-gene movement represented gene flow from small scale fields because the plots containing the marker were on average only 1/400th as large as fields supplying other outcross pollen. The outcrossing frequency for large fields (54.5) was nearly 10-times greater than that of research-sized plots (5.5). Since pollen load was the only difference between the two pollen sources that could account for these results, we must conclude that *detection* of pollen and gene flow is much more unlikely when the source is a small, research-sized plot. Unfortunately, most studies of trans-

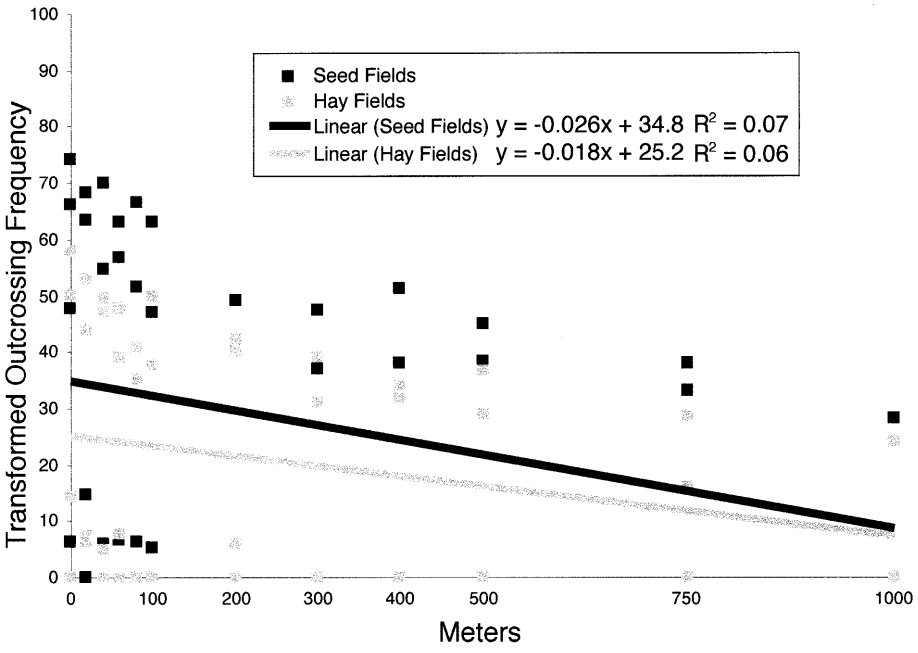

Fig. 6 Gene movement away from seed vs hay production fields. Data pooled across locations and field sizes indicate significant differences between field uses for outcrossing frequency (intercepts, P=0.00001), but not for rate of decline in frequency (slopes, P=0.1354)

gene dispersal and risk are done using small research-size plots of 10 m^2 or less.

Extrapolation of regression data beyond the range of independent variables used in a study is usually not advisable; however, in this study, extrapolation of outcrossing frequency to the point of zero will most likely provide an approximate *minimum* isolation distance for alfalfa. Solving for x in the regression formula (Fig. 5) for large fields gives a *minimum* isolation distance of 1557 m.

Pollen movement occurs not only from seed production fields, but also from alfalfa hay fields. Figure 6 shows that seed production fields have significantly ($P \leq 0.0001$) higher outcrossing rates (34.8), but hay fields also have significant gene flow (25.2). Regardless of the field type, the rate of decline in gene flow is not different (P=0.1354) between the field types.

Pollen movement between escaped plants

By examining widely dispersed, individual escaped alfalfa plants and their progeny using RAPD markers, gene movement among escaped alfalfa plants has been confirmed for distances up to 230 m (Table 1). The distribution of escaped alfalfa plants along roadsides in every state would most likely facilitate the escape and movement of transgenes placed into commercial alfalfa cultivars. Single escaped plants growing at considerable distances from alfalfa fields can contribute to medium- and long-distance gene spread away from fields and into escaped populations.

Individual trap plants placed in urban areas failed to set seed. These plants were at least 800 m from known plantings of alfalfa. The lack of self pollinations on these plants most likely indicated a very low level of pollinator activity, since the trap plants used are somewhat self fertile.

Table 1 Outcrossing frequencies, as determined by apparent non-self RAPD band patterns, on widely dispersed individual escaped alfalfa plants along roadsides in Prosser, Washington, and on individual plants planted in urban areas of Manhattan, Kansas and Prosser

Sample	Isolation distance (m)[a]	Total seeds tested	Outcrossing frequency
Escape #1	70	22	90.9
Escape #2	79	20	80.0
Escape #3	145	15	46.7
Escape #4	230	51	92.2
Urban #1	>800	0[b]	NA
Urban #2	>800	0	NA
Urban #3	>800	0	NA

[a] Distance to the nearest individual alfalfa plant or field
[b] No seed set in urban areas

These data suggest that complete containment of transgenes within alfalfa seed or hay production fields would be highly unlikely using current production practices. Containment within the production area may be facilitated if transgenic plants are grown in a field surrounded by non-transgenic plants. The border area would provide a buffer zone between the transgenic plants and the environment as a whole. Pollen carried from the transgenic plants, either by wind or insects, would most likely be deposited in the border population. In most cases, for insect-pollinated plants, the border area will be the same plant species as the transgenic plants, in order to ensure that the insect pollinators will not preferentially avoid the border area. This practice would also help guard against selection for resistance to transgenic insect resistance, such as cotton carrying the Bt gene, by providing buffer zones (refugia) of nontransgenic plants as

suggested by Tabashnik (1994). However, use of the same species will result in transgenic seed being produced on the border plants, necessitating the crop from the border plants be treated as transgenic.

Alternatively, it may be possible to substitute a different species in the border area, without losing the effectiveness of the buffering function. For example, surrounding transgenic alfalfa with Birdsfoot trefoil (*Lotus corniculatus* L.) most likely would buffer bee movement out of or into the transgenic field, but would result in no seed production on the Birdsfoot trefoil due to cross-incompatibility with alfalfa.

Since the likelihood for gene containment is small, environmental impacts, such as transgenic fitness, hybridization, competitive advantage, toxicology, and alerginicity, should be studied for all potential transgenes prior to release into the environment.

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