

Pollen-mediated transgene flow in the wind-pollinated grass species tall fescue (*Festuca arundinacea* Schreb.)

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Abstract

Information regarding gene flow in wind-pollinated, outcrossing forage grasses is essential for any future releases of value-added transgenic cultivars. Experiments on pollen dispersal was carried out by growing transgenic tall fescue (*Festuca arundinacea*) in a central plot, surrounded by exclosures containing recipient plants up to a distance of 200 m from the central source plants in eight directions. The central transgenic tall fescue plants carried a chimeric hygromycin phosphotransferase gene (*hph*) and a chimeric β -glucuronidase gene (*gusA*). Seeds were collected from the recipient plants and germinated seedlings were used for high throughput DNA isolation and polymerase chain reaction (PCR) analysis. More than 21,000 seedlings were PCR analyzed for the experiments conducted in three years. Transgenes were detected in recipient plants at up to 150 m from the central transgenic plot. The highest transgene frequencies, 5% at 50 m, 4.12% at 100 m and 0.96% at 150 m, were observed north of the central plot, the prevailing wind direction. Lower transgene frequencies were detected in other directions, particularly at 100 m and 150 m distances. No transgene was detected at 200 m distance in any direction. Transgene flow was less effective or ineffective when recipient plants were further away from the central donor plants. Southern blot hybridization analysis confirmed the transgenic nature of the PCR positive plants. A supplementary experiment demonstrated that transgene flow can be controlled by placing transgenic plantings downwind and long distances from non-transgenic seed increases, thus allowing tall fescue breeding and transgene development programs to be conducted concurrently at the same research station.

Introduction

Forages are the backbone of sustainable agriculture and contribute extensively to the world economy (Barnes and Baylor 1995). On a worldwide basis, grassland acreage is estimated to be twice that of cropland (Jauhar 1993). Most of the important forage and turf grasses are outcrossers, synthetic cultivars have been the main product of breeding for the majority of these species (Barker and Kalton 1989; Jensen et al. 1990). Thus, transgenic grasses pose a great challenge for risk assessment and biosafety studies.

Molecular breeding is being explored in forage grasses; transgenic plants have been obtained for several important species, such as tall fescue, red fescue, perennial ryegrass, Italian ryegrass and orchardgrass (Spangenberg et al. 1998; Wang et al. 2001a). Since wind-pollinated grass species have a high potential to pass their genes to adjacent plants, information regarding gene flow has become extremely important for any future release of value-added transgenic cultivars. Such information is not only essential for regulatory approval, but also needed for the adoption and management of transgenic grass cultivars. In the case of transgenic forage grasses, human consump-

tion is indirect. Risk assessment of transgenic forage grasses will likely focus on their environmental/ecological impacts and their safety to livestock. One of the main questions that needs to be addressed is the distance that grass pollen can disperse yet still remain viable and competitive enough for pollination. To date, there have been no reports on gene flow studies using transgenic plants in forage grasses.

Due to regulatory constraints, it has been extremely difficult to carry out field trials with outcrossing forage grasses in some countries. However, without doing the field experiments, many questions will remain unanswered. In the United States, field trials of transgenic grasses generally follow the standards for foundation seed production. The federal standards for cross-pollinated grasses (USDA CFR201.76), as well as those of several states (Oregon Seed Certification Service 2001; Nebraska Crop Improvement Association 1987), require an isolation distance of 274 m (900 feet) for foundation seed production. Isolation distances for registered seed and certified seed production are generally 91 m (300 feet) and 50 m (165 feet), respectively. There is no specific isolation standard for field trial or environmental release of transgenic grasses, although it is conceivable that pollen contamination levels will need to be much lower for transgenic versus conventional varieties. There are dozens of grasses that are cross-pollinated under natural conditions, thus current isolation standards are perhaps too general and broad. It is not clear if the isolation distance is sufficient to minimize contamination for either transgenic or non-transgenic seed production. Thus gene flow studies using transgenic plants will not only answer questions regarding isolation distance for growing transgenic grasses and future release of transgenic cultivars, but also provide valuable information for regulation of the current seed production system in outcrossing forage grasses.

Tall fescue (*Festuca arundinacea* Schreb.) is the most important forage species worldwide of the *Festuca* genus (Sleper and West 1996). It is a wind-pollinated, highly self-infertile allohexaploid forage species widely grown in southern Europe, east-central and southeast US and other parts of the world (Barnes 1990; Stadelmann et al. 1999). As an introduced species, tall fescue has become the predominant cool-season forage grass species in the US where it is grown on approximately 14 million hectares supporting over 8.5 million beef cows (Barnes 1990; Sleper and West 1996). Besides being used as forage, tall fescue is also widely used as turf in lawns, parks,

football fields, highway medians and roadsides. Tall fescue has been a major target species for genetic and agronomic studies (Jauhar 1993). In recent years, it has been used as a model system for large-scale sequencing of expressed sequenced tags (ESTs), gene discovery and genetic engineering (Wang et al. 2001a).

Transgenic tall fescue plants were generated by biolistic transformation of embryogenic suspension cells (Wang et al. 2003a). T1 and T2 progenies were obtained by crossing transgenic plants with seed-derived control plants (Wang et al. 2003a; Wang et al. 2003c). Here we report the use of T1 and T2 transgenic plants to investigate pollen-mediated transgene flow in tall fescue.

Materials and methods

Generation of transgenic tall fescue plants

Plant regeneration and genetic transformation systems have been developed for the most widely cultivated tall fescue cultivar, Kentucky-31 (Wang et al. 2003a). Sterilized seeds/caryopses of Kentucky-31 were used as explants for callus induction. Embryogenic calluses were individually transferred to liquid culture medium to establish single genotype-derived cell suspension cultures (Wang et al. 2003a; Wang et al. 1994). Cell clusters from the suspension cultures were used as direct targets for biolistic transformation to generate transgenic plants. A chimeric hygromycin phosphotransferase (*hph*) gene, which renders transformed cells resistant to hygromycin, was used as the selectable marker gene (Bilang et al. 1991). A chimeric β -glucuronidase (*gusA*) gene was co-transformed with the *hph* gene (Wang et al. 2003a). Hygromycin resistant calluses were obtained after microprojectile bombardment of suspension cells and subsequent selection in the presence of hygromycin (Wang et al. 2003a). Transgenic tall fescue plants were regenerated from the hygromycin resistant calluses and later transferred to the greenhouse and the field (Wang et al. 2003a; Wang et al. 2003c). Transgenic nature of the regenerated plants was confirmed by polymerase chain reaction (PCR), Southern and northern hybridization analyses and GUS staining (Wang et al. 2003a; Wang et al. 2003c).



Figure 1. Exclosures used for growing recipient non-transgenic plants. A) Overview of an exclosure, B) an exclosure with recipient plants.

Experimental design of pollen-mediated transgene flow

A preliminary experiment on pollen flow was carried out during 1999-2000. The experimental area was located in Ardmore, Oklahoma, USA. Mean annual temperature and rainfall of the experimental site were 17.7 °C and 912 mm, respectively. The general design included a central plot containing transgenic and non-transgenic plants that was surrounded by seed-derived non-transgenic recipient plants in eight directions (N, NE, E, SE, S, SW, W, NW) in 50 m increments up to 200 m distance from the central plot. The recipient plants were contained in square shape exclosures, with two recipient plants in each exclosure. An exclosure (Figure 1) is a 1.2 × 1.2 m pen constructed of welded wire and metal posts, and is used to exclude any grazing animals, such as deer or livestock, from the tall fescue plants. The central plot consisted of three replications of 16 primary transgenic plants, 16 cell suspension-derived plants and 16 seed-derived plants. All plants were transferred to the field in autumn of 1999. The field design in the central plot was mainly for comparing agronomic performance of transgenic plants with tissue culture- and seed- derived plants (Wang et al. 2003c). Due to the difficulties in finding a suitable piece of land for carrying out field trials with transgenic plants and the labor required for maintaining the experimental site to fulfill USDA regulations, the pollen flow experiment was combined with the investigation on agro-

nomous performance. Although it was realized later the central plot design was not very suitable for pollen flow study, much experience was gained from this preliminary experiment. All plants in the central plot were killed by spraying glyphosate herbicide prior to autumn, 2001.

Two more pollen flow experiments were carried out in 2001-2002 and 2002-2003, respectively. The experimental design (Figure 2) was improved based on the preliminary experiment. The central plot was planted with transgenic progenies (T1 and T2 plants) only. All the transgenic progenies were derived from one transformation event (T0). Transgenic T1 and T2 progenies were obtained by crossing transgenic plants with non-transgenic control plants under field (Wang et al. 2003a) and greenhouse conditions (Wang et al. 2003b). Transgenic nature of the T1 and T2 plants was confirmed by PCR and Southern hybridization analyses (Wang et al. 2003a; Wang et al. 2003b). Southern hybridization patterns were identical for the T0, T1, and T2 plants, and all the plants contained multiple copies of *hph* and *gusA*. In the year 2001-2002, forty-nine T1 and T2 transgenic plants were randomly arranged in a square central plot with plant-to-plant space of 0.61 m (Figure 2). The central plot was surrounded by exclosures containing seed-derived recipient plants. A total of 32 exclosures were built in eight directions with the distance between two exclosures being 50 m, and 4 exclosures in each direction (Figure 2). Each exclosure contained two plants. In order to avoid cross pollination between

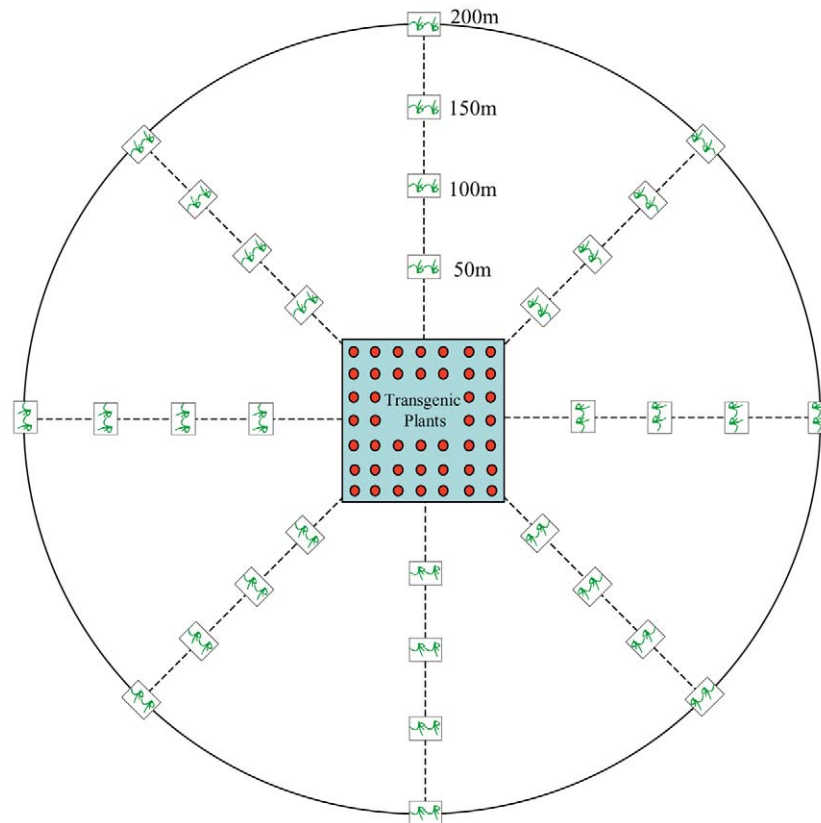


Figure 2. Field design of the pollen dispersal experiment. Central plot of transgenic plants was surrounded by exclosures containing recipient non-transgenic plants.

these two plants and to maximize the possibility of pollination by the central transgenic plants, one of the tillers was cut off if two tillers from the two plants flowered at the same time. Cutting tillers also promoted growth of late flowering or small tillers, thus extending the flowering period of the recipient plants and allowing a longer overlap with the flowering time of the central donor plants. The experiment was essentially repeated in the year 2002–2003, the only modification was that more transgenic progenies, with a total number of 225 T1 and T2 plants, were grown in the central plot as pollen donor.

Hygromycin selection of transgenic progenies

In order to test whether transgenic progenies could be simply identified by hygromycin selection, seeds harvested from three T1 transgenic plants were plated in petri dishes containing 0.8% (w/v) agar and lethal dose of hygromycin (200 mg/l). Control experiment was conducted by plating the seeds in petri dishes

with agar and without hygromycin. Germinated seedlings were PCR tested for the presence of *hph* gene.

DNA isolation and molecular analyses

Seeds harvested from the recipient plants were germinated in 96-cell (12×8) flats under greenhouse conditions ($390 \mu\text{E m}^{-2} \text{s}^{-1}$, 16 h light). The DNeasy 96 Plant kit (Qiagen #69181) was adopted for high-throughput purification of DNA from 3–5 week old seedlings; the DNA isolated by this method was mainly used for PCR analysis. Because the number of plants to be analyzed was very large, pooled leaf samples were collected from a row of young plants (mostly 12 plants, depending on germination) and used for DNA isolation. Two segments of approximately 1 cm long were sampled from each plant, and the pooled leaf material from the group of seedlings was collected into each of the collection microtubes in the DNeasy 96 plate. A metallic bead was added to each of the collection microtubes in the DNeasy 96

plate and the whole plate was frozen in liquid nitrogen. Rapid disruption of plant material was achieved by shaking the plate twice in a Mixer Mill MM300 (Qiagen #85110) at 30 Hz for 45 s, with orientation of the plate changed after the first shake. DNA was isolated by following the DNeasy 96 Plant Protocol from Qiagen (#69181).

Presence or absence of transgenes in the recipient plants was tested by PCR amplification of the coding regions of the transgenes. Following sets of oligonucleotide primers were used: *hph* 5'-CGCATAA-CAGCGGTCATTGACTGGAGC-3' (forward) and 5'-GCTGGGGCGTCGGTTTCCACTATCCG-3' (reverse); *gusA* AACAGTTCCTGATTAACCACAA-ACC-3' (forward) and 5'-GCCAGAAGTTCTTTTCCAGTACC-3' (reverse). The expected PCR products were 375 bp for *hph* and 634 bp for *gusA*. The total volume of reaction mixtures was 25 µl, including 1 µl genomic DNA (100-260 ng/µl), 0.5 µl of each primer (50 mM) and 22.5 µl Platinum PCR Supermix (Invitrogen #11306-016) and 0.5 µl H₂O. For positive control, 2 ng plasmid DNA was used instead of plant DNA. PCR was performed in PTC-200 DNA engine (MJ Research) in 96-well plates. Cycling parameters began with an initial hot start at 94 °C for 2 min, then 30 cycles of denaturation (94 °C; 40 s), annealing (60 °C; 30 s) and extension (72 °C; 1 min), followed by a final extension of 5 min at 72 °C. PCR amplification products were loaded into 96-well E-gel (Invitrogen # G7008-02) using a multi-channel pipettor and analyzed by electrophoresis. Once a positive band was identified from the pooled samples, DNA was isolated again from individual plants of that group, and the PCR positive plant in the group was identified and transferred to 11 cm pots.

For Southern hybridization analysis, total genomic DNA was isolated from freeze-dried leaf material of PCR-positive plants using the CTAB method (Lichtenstein and Draper 1985). Genomic DNA was digested with enzymes that cut out the coding region (*Bam* HI for *hph* gene construct, *Kpn* I for *gusA* gene construct). Undigested high molecular weight DNA was also loaded in the gel to confirm the stable integration of transgenes. Twenty microgram DNA was loaded in each lane. Gel electrophoresis and DNA blotting were carried out following standard protocols (Sambrook et al. 1989). Hybridization probes (*hph* and *gusA*) were [³²P] dCTP-labeled using the RadPrime DNA Labeling System (Invitrogen #18428-011), and the unincorporated nucleotides were removed by passing through the ProbeQuantTM.

G-50 Micro Columns (Amersham Pharmacia #27-5335-01). Southern hybridizations were performed using QuikHyb[®]. Hybridization Solution (Stratagene #201221) according to the manufacturer's specifications.

Containment of the transgenic material

To ensure the containment of the transgenic material, we followed a strict protocol during the entire experimental period to fulfill the performance standard for field trials under USDA notification. Special care was taken to make certain there was no inadvertent mixing or environmental release of the transgenic material. A well-contained vehicle was used to transport experimental material from the greenhouse to the field and from field to the laboratory. All field plants were recorded and monitored regularly. A strong electric fence was built around the center transgenic plot. Exclosures were constructed from metal posts and welded wire. All plants were planted and harvested by hand. Equipment was only used for seed cleaning and this was done in the laboratory. The equipment was inspected before and after the cleaning process. Any non-seed residues from the seed cleaning process were collected and autoclaved.

Results

The effectiveness of hygromycin selection was tested by plating seeds from T1 transgenic plants on agar plates containing hygromycin. Although half of the T1 seeds contained the transgene, only 4.7% of the seeds were able to germinate and producing viable seedlings in the presence of hygromycin. Control experiments revealed that 85% of the seeds germinated in the absence of hygromycin. Thus, germination rate of the seeds was substantially lower than the expected rate once selection pressure was applied. In addition, GUS staining revealed that only a small number of plants showing blue color in their leaves and the stainability varied with developmental stage. These results indicated that gene silencing occurred in the progenies; neither antibiotic screening nor GUS staining of seedlings was a reliable method to detect transgenes in the pollen flow study. Thus, PCR amplification of the transgenes (*hph*, *gusA*) was the main method for detecting the presence of transgenes from the harvested seeds. PCR positive plants were further confirmed by Southern hybridization analysis.

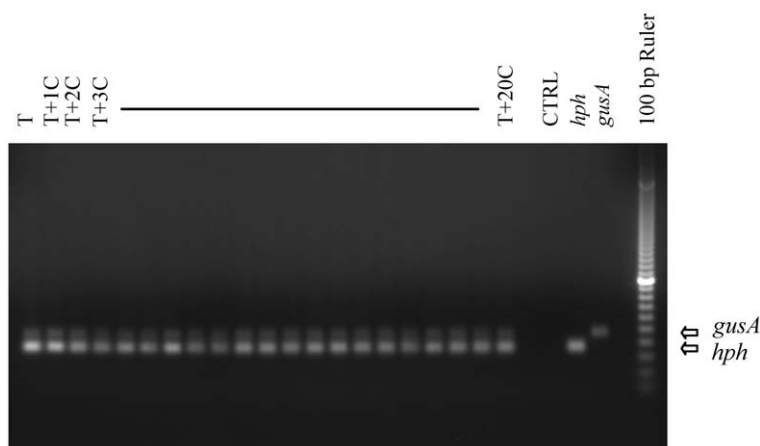


Figure 3. PCR analysis of DNA isolated from pooled samples of transgenic and non-transgenic materials. One piece of transgenic tissue was mixed with 1 – 20 pieces of control non-transgenic tissue, respectively. DNA was isolated from the mixed samples. T: transgenic tissue; T+1C: 1 piece of transgenic tissue mixed with 1 piece of control non-transgenic tissue; T+2C: 1 piece of transgenic tissue mixed with 2 pieces of control non-transgenic tissue, etc. Two sets of primers were used to amplify *hph* and *gusA* gene, respectively.

Seeds were harvested from the recipient plants in the exclosures and germinated in the greenhouse for testing the presence or absence of the transgenes. The use of pooled samples for the large-scale PCR screening saved a lot of time and chemical reagents. In order to make certain that the use of pooled samples did not compromise results, leaf material of a well-characterized transgenic plant was mixed with that of different numbers (1-20) of non-transformed plants, respectively. DNA was extracted from the pooled samples and amplified by PCR. The intensities of the amplified *hph* and *gusA* bands was similar between the samples, and the bands could be clearly seen for each sample (Figure 3), indicating that transgenes could be detected should one piece of transgenic material be present in a sample containing up to 20 pieces of non-transgenic tissue. In the actual large-scale DNA isolation and PCR screening work, pooled samples from up to 12 plants were used, with 2 segments (~1 cm) collected from each plant, to ensure that leaf material from each plant was sampled and subsequently ground. The amount of DNA obtained from the high throughput isolation procedure was approximately 90 μ l with a concentration of 100-260 ng/ μ l, which was more than enough for PCR analysis.

In the preliminary pollen flow experiment conducted in 1999–2000, the central plot consisted of primary transgenics, primary regenerants and seed-derived plants. Thus the pollen donors were both transgenic and non-transgenic plants, with more

plants of non-transgenic origin (seed-derived plants and primary regenerants from tissue culture). Nine hundred seedlings from the recipient plants in the exclosure at 50 m distance in the prevailing wind direction (north), as well as 400 seedlings respectively from north west and north east exclosures were analyzed. No positive plants were detected from seeds harvested from any of these exclosures, indicating that pollen from primary transgenic plants was not competitive enough to pollinate the recipient plants in the exclosures. This result was not surprising, considering the fact that the agronomic performance of the primary transgenic plants was generally inferior to the seed-derived plants. The primary transgenic plants had fewer tillers, a lower seed yield, and lower pollen viability (Wang et al. 2003c). Therefore, it is likely that non-transformed plants were the only pollen source in pollinating recipient plants in the exclosures.

Further agronomic evaluation revealed no major differences between the progenies of primary transgenic plants and seed-derived plants; transgenic progenies were fully fertile and their pollen viability was similar to that of seed-derived plants (Wang et al. 2004; Wang et al. 2003c). Therefore, transgenic progenies were suitable pollen donors for gene flow studies, and were exclusively grown in the central plot of the second and third experiments (Figure 2).

In the second experiment (2001–2002), a small number (49) of T1 and T2 plants was planted in the central plot as pollen donor. Seeds of recipient plants

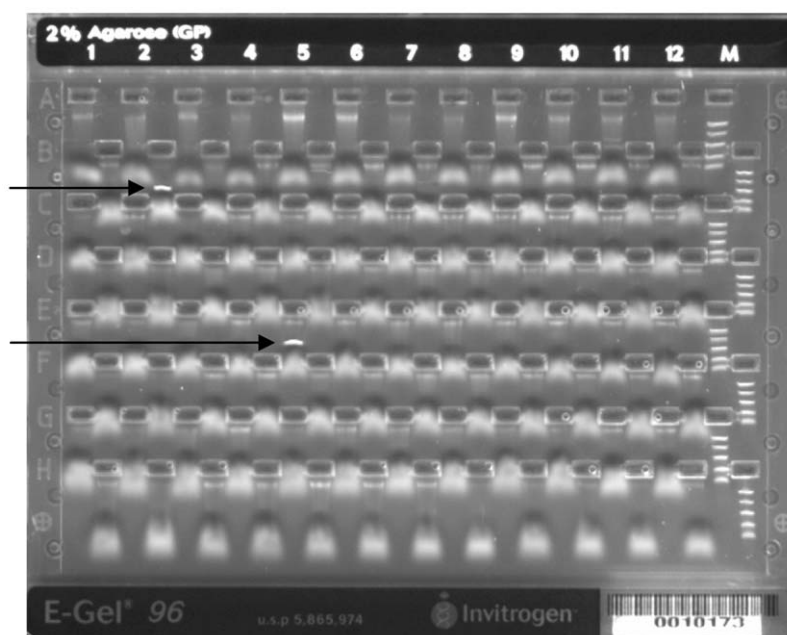


Figure 4. PCR of DNA isolated from progenies of recipient tall fescue plants grown in exclosures. Each DNA sample was isolated from a group of 6-12 seedlings; two positive groups of seedlings were detected in this plate. Arrow indicates the expected *hph* band. The upper band appearing in some lanes is high molecular weight genomic DNA.

from all 32 exclosures were germinated and used for high throughput DNA isolation. Depending on germination and the availability of seeds, 340–429 seedlings were sampled from each exclosure. DNA was extracted from more than 12,000 seedlings and used for PCR analysis. An example of PCR results using 96-well E-gel (Invitrogen #G7008-02) is shown in Figure 4. Because an ample amount of DNA was used and loaded in the very concentrated (2% agarose) E-gel, high molecular weight DNA was seen in most of the lanes (Figure 4). Although the presence of the expected 375 bp *hph* band in the gel was rare, it could be clearly distinguished from the high molecular weight DNA (Figure 4). Lanes containing the expected 375 bp *hph* band were considered PCR positive, indicating the presence of the transgene in the pooled sample. Because DNA used in the high throughput screening was isolated from a group of seedlings, once a positive band was detected, DNA was isolated from individual seedlings of that group. A second round of PCR using DNA from individual seedlings was carried out, and in all cases, one positive seedling was identified from the group. Since the *hph* and *gusA* primers were designed to amplify fragments with different sizes, two sets of primers were added to the same template in the second round of

PCR, and the transgenes (*hph* and *gusA*) were amplified simultaneously (Figure 5). This procedure not only saved time and chemicals, but also reduced the chance of obtaining false positives. Simultaneous amplification of *hph* and *gusA* in the seedlings indicated pollen mediated flow of both transgenes to the recipient plants. This result is as expected, because it has been shown that *hph* and *gusA* were closely linked or integrated into one chromosomal location in the primary transgenic plant and the two transgenes segregated together in both the T1 and T2 generations (Wang et al. 2003a).

Transgenes were detected in exclosures in different directions at 50 and 100 m distance from the central transgenic plants. The frequency of transgenes in the recipient plants in different directions (N, NE, E, SE, S, SW, W, NW) is shown in Table 1. At 50 m distance, transgenes were detected in recipient plants in N, NW, SE and SW directions with frequencies in the range of 0.25–0.73% (Table 1). At 100 m distance, transgenes were detected in N, NW and SE directions with respective frequency of 0.48, 0.24 and 0.24% (Table 1). The highest frequencies, 0.73% at 50 m and 0.48% at 100 m were observed north of the central plot. The prevailing wind during pollination was from south to north. No transgene was detected in any di-

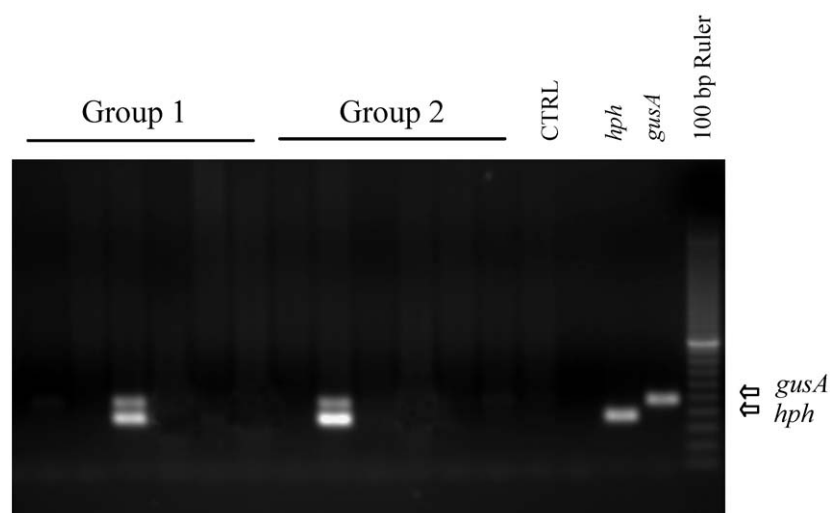


Figure 5. PCR of DNA isolated from the two positive groups of seedlings shown in Figure 4. Each DNA sample was isolated from individual seedlings in group 1 (six seedlings) and individual seedlings from group 2 (six seedlings). One positive seedling was detected from each group. Arrows indicate the expected *hph* and *gusA* bands, respectively.

Table 1. Frequency (%) of *hph* transgene in recipient tall fescue plants detected in the field experiment conducted during 2001–2002

Distance	N	NE	E	SE	S	SW	W	NW
50 m	0.73 (3/409)	0 (0/426)	0 (0/421)	0.25 (1/405)	0 (0/415)	0.48 (2/415)	0 (0/423)	0.27 (1/377)
100 m	0.48 (2/414)	0 (0/427)	0 (0/391)	0.24 (1/419)	0 (0/388)	0 (0/426)	0 (0/414)	0.24 (1/417)
150 m	0 (0/420)	0 (0/434)	0 (0/401)	0 (0/419)	0 (0/413)	0 (0/340)	0 (0/374)	0 (0/376)
200 m	0 (0/387)	0 (0/395)	0 (0/429)	0 (0/423)	0 (0/424)	0 (0/398)	0 (0/340)	0 (0/428)

Number of *hph* positive plants/number of tested seedlings is given in brackets. Forty-nine T1 and T2 transgenic plants were included in the central plot as pollen donor in the field experiment conducted during 2001–2002.

Table 2. Frequency (%) of *hph* transgene in recipient tall fescue plants detected in the field experiment conducted during 2002–2003

Distance	N	NE	E	SE	S	SW	W	NW
50 m	5.00 (12/240)	4.20 (2/48)	0.42 (1/240)	0.79 (2/253)	2.78 (8/288)	1.04 (3/288)	0.35 (1/288)	0.69 (2/288)
100 m	4.12 (8/194)	0 (0/233)	0 (0/46)	0.83 (2/240)	0.90 (2/223)	0 (0/311)	0 (0/137)	0.54 (1/184)
150 m	0.96 (2/209)	0.41 (1/245)	0 (0/84)	0.40 (1/253)	0 (0/72)	0 (0/255)	0 (0/220)	0.46 (1/219)
200 m	0 (0/125)	0 (0/248)	0 (0/219)	0 (0/48)	0 (0/193)	0 (0/284)	0 (0/83)	0 (0/205)

Number of *hph* positive plants/number of tested seedlings is given in brackets. Two hundred twenty five T1 and T2 transgenic plants were included in the central plot as pollen donor in the field experiment conducted during 2002–2003.

rection at 150 m and 200 m distances in this experiment.

In the third experiment (2002–2003), a relatively large number (225) of transgenic plants was planted in the central plot as pollen donor. Transgene frequencies and the actual number of plants analyzed are showed in Table 2. Approximately 6500 seedlings were analyzed in total for this experiment. Limited amount of seeds was harvested in some exclosures. Transgenes were detected in exclosures at 50, 100 and 150 m distances from the central plot. At 50 m dis-

tance, transgenes were detected in all directions with frequencies of 0.35–5%. Transgene frequencies were highest in direction N (5.0%), followed by NE (4.2%) and S (2.78%). At 100 m distance, transgenes were detected in N (4.12%), SE (0.83%), S (0.90%) and NW (0.54%). Relatively low transgene frequencies were also found at 150 m distance in N (0.96%), NE (0.41%), SE (0.40%) and NW (0.46%) directions. No transgenes were detected at 200 m distance in any directions (Table 2). The data from the second and third experiments clearly indicated that transgene flow was

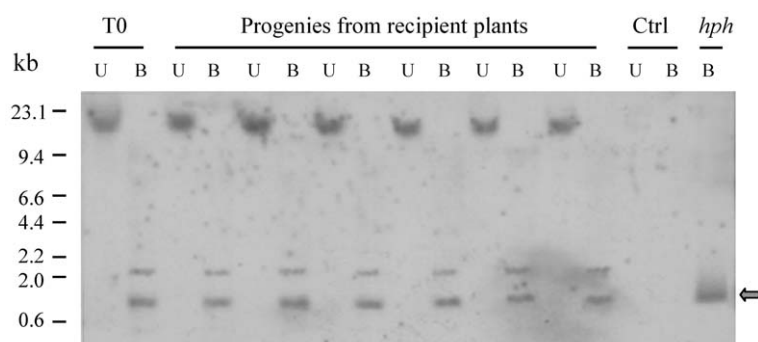


Figure 6. Southern blot hybridization analysis of PCR positive tall fescue plants using *hph* probe. T0: primary transgenic plants; Ctrl: untransformed plants serving as control, *hph*: plasmid control; U: undigested genomic DNA; B: genomic DNA digested with *Bam*H I, which excises the *hph* coding sequences in the plasmid used. Arrow indicates the expected size of the *hph* coding sequences after *Bam*H I digestion and Southern hybridization.

less effective or ineffective when recipient plants were further away from the central donor plants; plants in prevailing wind direction (north) had the highest possibility of being pollinated by the central transgenic plants. In the third experiment, transgenes were also detected at relatively high frequencies in the upwind direction (south) at 50 and 100 m distances from the central source of transgenic pollen.

Since PCR is a very sensitive method to detect transgenes, Southern blot hybridization analysis was used to confirm the transgenic nature of the PCR positive plants. Undigested high molecular weight DNA and genomic DNA digested with an enzyme (*Bam* HI for *hph*) cutting outside the coding sequences were blotted to nylon membrane after electrophoresis and hybridized with *hph* probe. Hybridization signals corresponding to high molecular weight bands and the *hph* coding sequences were observed in the Southern analysis (Figure 6). This analysis rules out any possibility of cross contaminations and verifies that the transgenes were stably integrated in the genome of the transgenic plants (Potrykus 1991). In addition, an identical hybridization pattern was observed in the primary transgenic plant and the progenies of recipient plants. Thus, Southern hybridization analysis confirmed that the PCR results were reliable and the data in the tables truly reflected transgene frequency.

Transgene control during field trials is required to prevent contamination of compatible plant populations that may be nearby. A supplementary experiment was carried out to verify that the transgene control measures were adequate for a tall fescue

breeding program located at an institution conducting field trials with transgenic tall fescue. Five different tall fescue polycross isolations were planted upwind, based on prevailing wind directions, from transgenic tall fescue plants. The isolations were located 889 to 2294 m from the transgenic material. Seed was harvested from the isolations on either a bulk or half-sib basis. Where possible, separate equipment was used to harvest and process seed from the transgenic plants; otherwise, seed from isolations was processed prior to seed from transgenic plants. DNA was isolated from an average of approximately 10 seedlings per half-sib family (121 families); 15 or more seedlings were sampled for the bulk seed increases. PCR was used to screen samples for the presence of the *hph* and *gusA* genes. Transgenes were not detected in either the half-sib families or the bulk seed increases. This was not unexpected given the large distances separating plantings and the direction of prevailing winds. The research demonstrated that transgenes can be controlled by placing transgenic plantings downwind and long distances from non-transgenic seed increases, and utilizing separate seed processing equipment. These measures have allowed tall fescue breeding and transgene development programs to be conducted concurrently at the same research station.

Discussion

Wind-pollinated forage and turf grasses can easily disperse pollen into surrounding populations. There are concerns regarding potential environmental and ecological problems associated with release of trans-

genic grasses, such as invasion and disruption of natural ecosystems, extinction of native plants or increased weediness. Since many of the potential problems begin with transgene flow or transgene escape, a logical first step in biotechnology risk assessment is to analyze and quantify the extent of pollen dispersal.

Traditional methods such as pollen trap, isozyme and morphological markers, have been used to study pollen flow in a few species. Pollen traps were used to collect pollen from non-transgenic perennial ryegrass (Giddings et al. 1997a; Giddings et al. 1997b). The maximum distance collected was 80 meters. Ryegrass pollen was found at 80 m distance from the source plants, although the amount of pollen collected was much less than that near the center ryegrass field. There are inherent problems with the pollen trap method, since it is impossible to know whether the collected pollen still remains viable and competitive enough to pollinate plants, and it is almost impossible to distinguish pollen from one grass species from another or from cereals. An isozyme marker (*Pgi-2*) was used to investigate pollen dispersal in non-transgenic meadow fescue (Nurminen et al. 1998; Rognli et al. 2000). Pollen was captured by recipient plants at a distance of 250 m. However, the isozyme allele was also present in the surrounding areas of the experimental plot (Rognli et al. 2000). Morphological characters can be influenced by the environment and the identification of such traits can be subjective. The use of morphological markers has produced conflicting results. Pollen contamination in perennial ryegrass by an outside pollen source has been reported to be as little as 1% at 7.3 m (Copeland and Harding 1970), or as much as 10% at 182 m (Griffiths 1951). In smooth brome grass, Johnson et al. (1996) reported pollen contamination of less than 5% between 22 and 27 m. Due to the lack of reliable morphological markers, there have been no reports on pollen flow in tall fescue.

Transgenic plants provide unique material to study gene flow in grasses, and only by using transgenic material can research realistically reflect the situation concerning any future release of transgenic grass cultivars. The detection of transgenes by molecular techniques offers distinctive advantages over the use of morphological or biochemical markers by providing clear-cut information on presence or absence of transgenes without subjective judgment or environmental influence.

Tremendous progress has been made in genetic transformation of forage and turf grasses in the past

decade. Transgenic plants have been produced in tall fescue (Bettany et al. 2003; Bettany et al. 1998; Chen et al. 2003; Dalton et al. 1995; Dalton et al. 1998; Kuai et al. 1999; Spangenberg et al. 1995a; Spangenberg et al. 1995b; Wang et al. 2003a; Wang et al. 1992; Wang et al. 2001b), meadow fescue (Spangenberg et al. 1995a), red fescue (Altpeter and Xu 2000; Spangenberg et al. 1994; Spangenberg et al. 1995b), perennial ryegrass (Altpeter et al. 2000; Dalton et al. 1998; 1999; Spangenberg et al. 1995c), Italian ryegrass (Bettany et al. 2003; Dalton et al. 1998; 1999; Wang et al. 1997; Ye et al. 1997), creeping bentgrass (Asano et al. 1998; Lee 1996; Liu et al. 1998; Yu et al. 2000), orchardgrass (Cho et al. 2001; Denchev et al. 1997; Horn et al. 1988), switchgrass (Richards et al. 2001), bahiagrass (Smith et al. 2002) and blue grama grass (Aguado et al. 2002). In addition to the development of transformation methods, transgenic grasses have been generated with potentially useful agronomic genes, e.g., herbicide resistant transgenic creeping bentgrass, tall fescue and red fescue (Asano et al. 1998; Lee 1996; Liu et al. 1998; Spangenberg et al. 1994; Wang et al. 1992; Yu et al. 2000), transgenic Italian ryegrass with altered fructan accumulation (Ye et al. 2001), transgenic perennial ryegrass with down-regulated pollen allergens (Spangenberg et al. 2001; Wu et al. 1997), transgenic tall fescue expressing sulphur-rich sunflower albumin genes for improved nutritional quality (Wang et al. 2001b), virus resistant transgenic perennial ryegrass (Xu et al. 2001), fungal disease resistant creeping bentgrass (Dai et al. 2003; Guo et al. 2003) and improved forage digestibility by transgenic manipulation of lignin biosynthesis in tall fescue (Chen et al. 2003).

In contrast to the many laboratory studies on transgenic grasses, there have been very few field tests of these transgenic plants, partially due to regulatory hurdles for these outcrossing species in some countries. Risk assessment of transgenic forage and turf grasses, particularly for forage-type grasses, has been far lacking behind that of cash crops such as corn, soybean, canola (Conner et al. 2003; Messeguier 2003). To date, the only reported studies on pollen flow have been on transgenic creeping bentgrass (Belanger et al. 2003; Wipff and Fricker 2001). Herbicide resistant progenies of creeping bentgrass were recovered at 292 m maximum distance from the source transgenic plants (Wipff and Fricker 2001). Belanger et al. (2003) measured frequencies of inter-specific hybridization between transgenic creeping

bentgrass and four related species in a hexagonal design, with maximum distance of 15 m from the central transgenic plants. It was convincingly documented that interspecific transgenic hybrids were recovered between creeping bentgrass and *Agrostis capillaris* and *A. castellana* at frequencies of 0.044 and 0.0015%, respectively (Belanger et al. 2003). It should be noted that creeping bentgrass is a slow growing grass species exclusively used for turf purposes. This means that, unlike other more aggressive grasses, herbicide resistance (e.g., Roundup Ready® bentgrass) is not a major biosafety concern for this species. However, the results of gene flow studies in creeping bentgrass may not be directly applied to other major forage and turf species, such as fescues and ryegrasses. Thus, there is an urgent need for gene flow studies in major forage grasses for the future deregulation and release of value-added transgenic cultivars.

As the first effort to investigate pollen-mediated transgene flow in tall fescue, the present study used transgenic progenies as a pollen source, surrounded in eight directions by exclosures containing recipient non-transgenic plants. Hygromycin selection of transgenic seeds/seedlings revealed that gene silencing occurred and inactivated the expression of transgenes in the progenies. In the last decade, gene silencing has been recognized to be a general phenomenon reported for many species transformed with a variety of chimeric genes (Grant 1999; Kunz et al. 2001). Epigenetic silencing may persist over many cell divisions or plant generations (Paszkowski and Whitham 2001). Therefore, even though antibiotic or herbicide selection may be an effective method in some cases, the seeds or seedlings from the recipient plants need to be tested by molecular analysis to make certain that the transgene(s) segregates in an expected manner, and the phenotype selection is closely correlated with the presence of transgenes. In other cases where gene silencing occurs, a simple antibiotic or herbicide selection method will produce misleading results. The most reliable method is to detect the presence of transgenes by molecular methods, such as PCR and Southern hybridization analysis.

High throughput DNA isolation and PCR screening methods were developed in this study; these techniques are essential for pollen flow studies in grasses. A large number of seedling from recipient plants in the exclosures were analyzed by PCR and the positive ones were confirmed by Southern hybridization analysis. When non-transformed plants and primary

transgenics were grown together in the central plot in the preliminary study, no transgenes were detected in the exclosures. By utilizing only transgenic T1 and T2 plants as the exclusive source of transgenic pollen in the second experiment, transgenes were detected up to 100 m distance from the central transgenic plants; the highest frequency (0.48%) was detected in the prevailing wind direction. In the upwind direction, no transgenes were detected at even 50 m distance from the central transgenic plants. Compared with the third experiment, the number of transgenic plants used in the second experiment was relatively small. With a relatively large number of transgenic plants included in the third experiment, transgenes were detected at higher frequencies and at longer distances when compared with the second experiment. Highest frequency of up to 5% was detected at 50 m distance in the prevailing wind direction. Transgenes were also found at a frequency of up to 0.96% at 150 m distance. No transgene was detected when the exclosures were 200 m away from the central transgenic plants. The data indicated that the abundance of transgenic pollen was an important factor affecting transgene flow. Since flowering time of tall fescue plants spanned more than a month, weather conditions may have also played an important role on frequencies of transgenes in the recipient plants. In the upwind direction(s) of the third experiment, the third highest transgene frequency (after N and NE) was observed at 50 m distance, and transgenic progenies were still detectable at 100 m distance; the observation coincided with the fact of frequent change of wind directions during the flowering season. Thus the wagon wheel design was very appropriate for studying pollen flow in grasses. Although the abundance of transgenic pollen affected the effectiveness of transgene flow, a balance between the potential outcome and the feasibility of the field test has to be considered when designing an experiment. The use of excessively large numbers of transgenic plants may not drastically increase transgene frequency in the recipient plants; it has been found that in large-scale plantings of non-transgenic grasses, pollen from the border plants played a major role in pollinating recipient plants (O.A. Rognli, personal communication).

Results from the three experiments revealed that the maximum effective travel distance of transgenic pollen was 150 m, indicating that for transgenic field trials of tall fescue, the isolation distance required by USDA (300 m) is enough to prevent transgene flow to neighboring plants, at least for small-scale field tri-

als. The isolation requirement might be applicable to countries with more stringent regulations on environmental releases of transgenic grasses. Our research also demonstrated that growing transgenic plants in the down wind direction will not affect conventional breeding activities in the upwind direction at sufficient isolation distance.

Some lessons have been learned from the field experiments, including (1) only fertile transgenic progenies should be used in a gene flow study, (2) sturdy exclosures should be constructed, since animals have a tendency to rub on the exclosures, (3) a longer overlap of flowering time of grasses can be partially controlled by cutting back tillers or by application of fertilizers, and (4) reserve plants should be vernalized and maintained to replace any dead plants in the spring. One or more years could be lost if these problems are not considered at the beginning of an experiment.

In the context of risk assessment, the use of herbicide resistance in forage and turf grasses should consider other production systems. Glyphosate resistance is one of the first traits commercialized in transgenic crops; Roundup Ready® corn, cotton and soybean cultivars have been widely adopted in recent years. In grasses, the first naturally occurring glyphosate-resistant biotype of *L. rigidum* was identified in Australian orchards due to the continuous application of glyphosate herbicide (Baerson et al. 2002; Lorraine-Colwill et al. 2001). There have been many studies devoted to this new biotype since its discovery in 1996, and glyphosate resistance in this species has been considered as a major emerging threat to current weed management practices in Australia (Baerson et al. 2002; Lorraine-Colwill et al. 2001). Therefore, even though glyphosate resistance can be a beneficial trait in forage and turf grasses, we need to be extremely careful in employing this trait in aggressive grass species.

Pollen-mediated gene flow is not unique to transgenic plants, it is a natural process that has happened in the past and will continue to happen in the future. The introduction of molecular techniques has brought new attention to the process and raised both economic and ecological issues for scientists and policymakers to consider. As novel transgenic grasses are tested and grown, preventing unwanted gene flow will present technical and regulatory challenges as well as possible economic conflict. Research on gene flow of outcrossing grasses not only involves the already complicated biological systems, but also highly vari-

able environmental conditions. Because many of the important forage and turf species are widely grown and highly adaptable to marginal areas, it is simply impossible to mimic all the situations that might happen under natural conditions. Therefore, a future study to reveal a worst case scenario will be very meaningful. Such a study could take advantage of the outcrossing nature of grasses by using single genotype-derived plants as recipients in the exclosures. Plants of single genotype origin (obtained by vegetative propagation of a single plant) will not pollinate each other. This will maximize the possibility for transgenic pollen to pollinate recipient plants, and minimize the possibility of pollination within and between the exclosures. Thus the proposed study should reveal the maximum pollination distance of transgenic pollen, a worst case scenario that could happen under natural conditions. In addition, by including more exclosures in the experiment, precise modeling of transgene flow will provide valuable information for different occasions. Collaborative efforts at the national and international levels are needed to carry out research in this complex area to accumulate enough scientific knowledge for the deregulation of transgenic grass species.

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