AN ABSTRACT OF THE DISSERTATION OF

Maria Luz Zapiola for the degree of Doctor of Philosophy in Crop Science presented on October 22, 2009.

Title: Gene Flow from Transgenic Glyphosate-Resistant Creeping Bentgrass (*Agrostis stolonifera* L.) at the Landscape Level.

Abstract approved:

Carol A. Mallory-Smith

Creeping bentgrass (*Agrostis stolonifera* L.) is an outcrossing, small-seeded, perennial grass that can establish outside of cultivation and has several compatible relatives. Glyphosate is a nonselective, broad spectrum, herbicide. Transgenic glyphosate-resistant (GR) creeping bentgrass (GRCB) was developed by The Scotts Company and Monsanto, but is still under USDA-APHIS regulated status. In 2002, 162 ha were planted to GRCB within a 4,500 ha control area north of Madras, OR, and produced seed in 2003. After a wind event moved swathed panicles off the GRCB fields, the fields were taken out of production and a mitigation program was initiated. The goal of this study was to assess the potential of gene flow from GRCB at the landscape level. A four-year survey was conducted *in situ* to determine the proportion of GR plants established outside of cultivation. Evidence of gene flow was found in all years. In 2006, despite the ongoing mitigation program, 62% of the 585 creeping bentgrass plants tested *in*

situ were GR. Panicles were collected from *Agrostis* spp. and rabbitfoot grass (*Polypogon monspeliensis* (L.) Desfontaines) plants for the four years. Seedlings produced were screened in the greenhouse using glyphosate to assess the occurrence of pollen-mediated gene flow. Gene flow via pollen was found in all four years. A set of chloroplast microsatellite (cpSSR) markers and a *matK* indel marker were developed to aid in the identification of *Agrostis* spp. and potential hybrids. Chloroplast markers were used in combination with nuclear ITS sequence to confirm transgenic interspecific and intergeneric hybrids produced *in situ*. The effect of soaking time and water temperature on seed germination potential was studied to explore the potential of seeds-mediated gene flow in time and space. Creeping bentgrass seeds did not lose their germination after 17 wk in water at 20 C and germination was 46% after 17 wk at 4 C. Panicles were found to travel in an irrigation canal at an average rate of 19 ± 1 m min^{-1}. Results of this study should be used in the decision making process for authorization of field trials and deregulation of transgenic crops, especially grasses.

Gene Flow from Transgenic Glyphosate-Resistant Creeping Bentgrass (*Agrostis stolonifera* L.) at the Landscape Level

by
Maria Luz Zapiola

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APPROVED:

Major Professor, representing Crop Science

Head of the Department of Crop and Soil Science

Dean of the Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Maria Luz Zapiola, Author

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CONTRIBUTION OF AUTHORS

Dr. Carol A. Mallory-Smith advised all aspects of the research conducted and provided valuable feedback throughout the project. She also was actively involved in the preparation and improvement of the manuscripts. Ms. Claudia Campbell provided invaluable help during the *in situ* survey and global positioning system data analysis. She also was involved in the improvement of the manuscript. Mr. Marvin Butler was a valuable support during the in situ survey and contributed with edits to the manuscript. Dr. Rich C. Cronn proposed and advised the development of the chloroplast molecular markers, and provided valuable suggestions to the manuscript.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

Page

LIST OF FIGURES

LIST OF TABLES

DEDICATION

To my parents Marité and Hugo who always encourage me to chase my dreams.

Gene Flow from Transgenic Glyphosate-Resistant Creeping Bentgrass (*Agrostis stolonifera* L.) at the Landscape Level

CHAPTER 1: GENERAL INTRODUCTION

Transgenic crops have had widespread adoption worldwide since their introduction in the mid 1990's, with 125 million ha planted in 2008 in 25 countries around the globe (James 2008). Of all crops and traits, herbicide resistance has been consistently the dominant trait adopted. One of the advantages of herbicide resistant transgenic crops, such as glyphosate-resistant (GR) crops, is the simplification of weed control.

Glyphosate is a broad-spectrum, nonselective, systemic, herbicide that inhibits the 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) enzyme in the chloroplast, which is part of the pathway of aromatic amino acids in the plant, and therefore, limits the synthesis of proteins resulting in the death of the plant. Glyphosate is very effective in controlling many species of annual and perennial weeds, and can be safely sprayed over GR crops at different stages of development. Therefore, weed control in GR crops is more flexible and potentially less expensive than previous herbicide options (Dill 2005; Gianessi 2005).

Creeping bentgrass (*Agrostis stolonifera* L.), a fine-textured stoloniferous perennial turfgrass, is the most widely used cool-season grass for high-quality golf course tees, greens and fairways throughout the world (Turgeon 1996; Warnke 2003). The increasing importance and value of turfgrass is reflected in the size of the turfgrass seed market with annual sales between $580 million to1.2 billion

(Wipff & Fricker 2001). Control of grass weeds in golf courses is problematic due to the lack of selective herbicides (Gange et al. 1999).

Glyphosate-resistant creeping bentgrass cultivars could simplify and reduce the cost of weed management in golf courses. The Scotts Company and Monsanto developed transgenic GR creeping bentgrass (GRCB). The Roundup Ready® trait conferred by the *CP4 EPSPS* gene, originally from *Agrobacterium tumefaciens* strain CP4, that encodes a glyphosate-resistant form of the plant's EPSPS was engineered via recombinant DNA methods into the creeping bentgrass cultivar 'Backspin'. The event was designated ASR368 and, even though a petition for deregulation of GRCB was submitted in April 2003, GRCB is still under USDA-APHIS regulated status.

The concerns regarding the ecological impact of the adoption of genetically engineered (GE) organisms are related, in general, to the phenotypic traits associated with the introduced gene, the biology of the transformed species, and the presence of wild relatives as they can play a critical role in gene flow (Wipff & Fricker, 2001). The possible transgene escape to feral plants, related species, and non GE crops raises ecological and commercial concerns (Luo et al. 2005). Marvier & Van Acker (2005) emphasized the fact that it is unlikely transgenes can be retracted once they have escaped from the crop. Pollen flow and gene introgression into local populations are two critical events for gene transfer to occur, and they have to be addressed to ensure environmentally and agriculturally safe introduction of GE crops (Wipff & Fricker, 2001). However, gene flow via crop-weed hybridizations is not unique to genetic engineering as it

also occurs with commercially breed crops (Ellstrand, 2003a,b; Lu & Snow, 2005). Creeping bentgrass is predominantly wind cross-pollinated and usually highly self incompatible resulting in a high outcrossing potential.

The prospective commercialization of GRCB cultivars in particular, raised questions about the potential for pollen-mediated gene flow to non-transgenic creeping bentgrass crops or other compatible *Agrostis* spp.- redtop (*A. gigantea*), colonial bentgrass (*A. capillaris*), dryland bentgrass (*A. castellana*), velvet bentgrass (*A. canina*), spike bentgrass (*A. exarata*) - and rabbitfoot grass (*Polypogon* spp.). There also have been concerns of “contamination” of non-genetically modified crops with GRCB seed due to commingling, which could result in the lost of international markets that have zero tolerance for transgenic organisms. Another concern was the difficulty to control volunteer GRCB.

In 2002, the Oregon Department of Agriculture established a seed production control area in central Oregon and allowed the planting of 162 ha of transgenic GRCB to start increasing seed. The 162 ha were planted in eight different fields within the control area. Creeping bentgrass has been grown for seed in the Madras area in the past, which contributed to the adventitious presence of the species in the area where redtop is also abundant. This situation presented a unique opportunity to perform an *in situ* study of gene flow at a landscape level.

The objectives of the project were: 1) evaluate the frequency of GR *Agrostis* spp. among the plants found in irrigation canals, ditches and ponds in the Madras area; 2) assess the degree of pollen-mediated gene flow at the landscape

level; 3) develop molecular markers that could aid in the identification of the *Agrostis* species and potential hybrids; and 4) evaluate the potential of gene flow via seeds.

We conducted a 4 year survey where we tested creeping bentgrass and compatible species for the presence of the transgene. Panicle and leaf tissue samples were collected. Panicles were threshed, and the seeds were used to perform herbicide screening experiments in the greenhouse to determine the proportion of gene flow via pollen at the landscape level. Simultaneously, other gene flow studies outside the control area were conducted by Watrud et al. (2004) and Reichman et al. (2006). Due to the difficulty in identifying the different *Agrostis* species and the potential interspecific hybrids based solely on morphology, we developed a *matK* indel and chloroplast microsatellite (cpSSR) markers to combine with analysis of nuclear ITS sequences to aid in species determination. Because there was a significant difference between the percentage of GR plants found *in situ* and the percentage of GR seedlings obtained from the greenhouse screening, we evaluated the potential of seeds to be a means of gene flow. A study was performed to determine the impact of soaking time and water temperature on creeping bentgrass germination potential and assess how fast a panicle could be carried away in an irrigation canal in central Oregon.

The final goal of this study was to better understand the potential of gene flow from GRCB at the landscape level. Results of this study should be used in the decision making process for authorization of field trials and deregulation of

transgenic crops, especially outcrossing, wind-pollinated, small-seeded, perennial transgenic grasses.

CHAPTER 2: ESCAPE AND ESTABLISHMENT OF TRANSGENIC GLYPHOSATE-RESISTANT CREEPING BENTGRASS (*Agrostis stolonifera*) IN OREGON, USA: A FOUR YEAR STUDY.

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SUMMARY

Gene flow from transgenic crops to feral populations and naturalized compatible relatives has been raised as one of the main issues for the deregulation of transgenic events. Creeping bentgrass *Agrostis stolonifera* L. is a perennial, outcrossing grass that propagates by seeds and stolons. Transgenic Roundup Ready® glyphosate-resistant creeping bentgrass (GRCB), which is currently under USDA-APHIS regulated status, was planted in 2002 on 162 ha within a production control area in Oregon, USA. We conducted a study to assess transgene flow from the GRCB fields. A survey within and around the production control area was performed during the year that the GRCB fields produced seed and for 3 years after the fields were taken out of production. Transgene flow was determined by testing creeping bentgrass and its relatives for the expression of the glyphosate resistance transgene. While GRCB seed production practices were strictly regulated, evidence of transgene flow was found in all years. In 2006, three years after the transgene source fields were taken out of production and a mitigation program was initiated, 62% of the 585 creeping bentgrass plants tested *in situ* were glyphosate-resistant (GR). Our results document not only the movement of the glyphosate resistance transgene from the fields, but also the establishment and persistence of high frequencies of GR plants in the area, confirming that it was unrealistic to think that containment or eradication of GRCB could be accomplished.

Synthesis and applications: These findings highlight the potential for transgene escape and gene flow at a landscape level. The survey provides empirical frequencies that can be used to design monitoring and management methods for genetically engineered varieties of outcrossing, wind-pollinated, perennial grasses and to evaluate the potential for coexistence of GE and non-GE grass seed crops. Such information should also be used in the decision making process for authorization of field trials and deregulation of GE events.

Key-words: Agrostis, coexistence of GE crops, CP4 EPSPS, gene flow, genetically modified crops, genetic engineering, glyphosate resistance, glyphosate-resistant crops, transgenic crops.

INTRODUCTION

Gene flow from genetically engineered (GE) crops has been raised as a major concern when considering the deregulation of new GE events in general (Giddings 2000; Ellstrand 2001, 2003a,b; Andow & Zwahlen 2006). Gene flow is defined as the change in gene frequency due to movement of gametes, individuals or groups of individuals from one place to another (Slatkin 1987).

Creeping bentgrass *Agrostis stolonifera* L. is the most widely used cool-season grass for high quality golf course tees, greens and fairways (Turgeon 1996; Wipff & Fricker 2001; Warnke 2003), where other grasses are the main weed problem (Gange, Lindsay & Ellis 1999; Belanger *et al*. 2003a). Glyphosate is a broad spectrum, nonselective herbicide; therefore, transgenic glyphosate-resistant creeping bentgrass (GRCB) would allow a more flexible and effective way of controlling problem weeds.

The *CP4 EPSPS* gene, isolated originally from *Agrobacterium* sp. strain CP4, which encodes the CP4 EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase) protein that confers resistance to glyphosate herbicide, was incorporated via DNA recombination techniques into a creeping bentgrass cultivar (event ASR368, The Scotts Company, Monsanto Company). Although a petition (APHIS petition no 03-104-01p) was submitted in April 2003 requesting the deregulation of Roundup Ready® GRCB, this GE grass is still under USDA-APHIS regulated status. Glyphosate-resistant creeping bentgrass was the first GE crop in the USA for which an Environmental Impact Statement was requested.

The main concerns raised regarding the deregulation of GRCB were the contamination of non-GE grass seed crops (especially of seed lots to be exported to countries with zero tolerance for GE organisms), the introgression of the *CP4 EPSPS* gene via pollen flow into feral and naturalized compatible weed species and the control of GRCB volunteer plants. Another concern was the evolution of glyphosate-resistant weeds due to the increased selection pressure of repeated glyphosate applications on GRCB fields.

Even though creeping bentgrass is a cosmopolitan, phenotypically plastic and evolutionarily adaptive species, it is rarely invasive in natural or semi-natural areas (MacBryde 2006). Nevertheless, creeping bentgrass has several characteristics that increase the potential of transgene flow, establishment, introgression and persistence into nearby compatible sympatric populations. First, it is an outcrossing, wind-pollinated, perennial grass that propagates sexually by seed and vegetatively by stolons. Second, seeds of creeping bentgrass are small, approximately 13,500 seeds g^{-1} (AOSA 2002), and thus can be easily dispersed. Creeping bentgrass seed can germinate soon after dispersal but also persist in the seed bank where they germinate for at least 4 years (unpublished data). The average seed yield for creeping bentgrass in Oregon is 600 kg ha^{-1} (USDA-NASS 2006), representing 8.1 x 10^9 seeds ha^{-1}. Finally, creeping bentgrass is an allotetraploid, and polyploidy is generally associated with a greater probability of producing fertile interspecific hybrids (Warnke 2003).

Agrostis, one of the most difficult and complicated grass genera from a taxonomic point of view, is a cosmopolitan genus with approximately 200 species

worldwide that exists in a broad variety of habitats (Hitchcock 1971; Hitchcock & Cronquist 1973; Harvey 1993; Warnke 2003). Creeping bentgrass, redtop *A. gigantea* Roth, colonial bentgrass *A. capillaris* L., dryland bentgrass *A. castellana* Boiss. & Reuter, velvet bentgrass *A. canina* L. and brown bentgrass *A. vinealis* Schreber form a complex of interpollinating, cross-compatible species. Sympatric populations may contain interspecific hybrids with varying degrees of pollen fertility and seed set (Wipff & Fricker 2001; Belanger *et al.* 2003a). Redtop, colonial, dryland and velvet bentgrass, as well as spike bentgrass *A. exarata* Trin., occur in Oregon. Creeping bentgrass has also been reported to form intergeneric hybrids with rabbitfoot grass *Polypogon monspeliensis* (L.) Desfontaines and water bent *P. viridis* (Gouan) Breistr., both of which occur in Oregon (Wipff & Fricker 2001).

In 2002, the Oregon Department of Agriculture established a 4,500 ha seed production control area at Jefferson County, 2.5 km north of Madras (44° 38' 1.00" N, 121° 7' 41.99" W), Oregon, USA (Oregon Administrative Rule -OAR-603-052-1240), and 162 ha were planted with GRCB. The control area is an elevated plateau with an elevation of about 720 m and has a 270 m drop to the Deschutes River to the NW and a more moderate drop to Mud Springs Creek to the E. The other limits are mainly local roads on the NE, and USA Highway 26 on the SW. The region is characterized by a high desert climate with average maximum and minimum temperatures of 31° and 7°, and 6° and -6°C, for July and January, respectively. The average rainfall in the area is 246 mm per year. However, because the control area is part of the North Union Irrigation District, it

has mesic areas, such as banks of canals, irrigation and drainage ditches, and ponds, that are ideal for establishment of creeping bentgrass seedlings. The main crops planted are Kentucky bluegrass *Poa pratensis* L., rough bluegrass *Poa trivialis* L.*,* carrot *Daucus carota* L. ssp. *sativus* (Hoffmann) Arcang., onion *Allium* spp. and garlic *Allium sativum* L., all for seed production, and alfalfa *Medicago sativa* L. for hay. The control area has multiple growers, each with their own weed management program. The surrounding area consists of grasslands and arid landscapes to the NW and E, and irrigated agricultural land to the S.

The 162 ha of GRCB planted in 2002 were distributed among eight fields within the control area. In 2003, after seed harvest, the eight GRCB fields were taken out of production. An additional 2.4 ha GRCB field was planted in 2003, flowered and produced seed in 2004. Production practices within the control area were strictly regulated and monitored to minimize gene flow from GRCB seed fields. Some of the control area requirements were: 1) non-GE *Agrostis* spp. could not be planted, grown, or handled within the control area; 2) GRCB was to be located more than 400 m away from any bentgrass field outside the control area; 3) field borders, ditch banks, and roadsides within 50 m of the GRCB fields were to be kept free of *Agrostis* spp.; 4) waterways leaving GRCB fields were to be kept free of *Agrostis* spp. for 50 m; 5) GRCB seed was to be transported in enclosed containers; 6) equipment, such as swathers and balers, had to be thoroughly cleaned before leaving the control area; combines were to be used exclusively for GRCB and thoroughly cleaned and fumigated after use; 7) all straw from GRCB fields was to be burned within the control area or processed in a way

that devitalized the creeping bentgrass seeds; 8) GRCB stands were to be removed by application of effective herbicide after watering to promote growth, followed by shallow tillage; 9) GRCB volunteers were to be controlled in the subsequent crop (Oregon Administrative Rule -OAR- 603-052-1240). At the time of seeding, The Scotts Company further increased the distance from the GRCB fields to be kept free of *Agrostis* spp. to 300 m. Although not part of the original control area rules, fields were fumigated with metham sodium after the removal of the GRCB stands.

Knowledge of the extent of transgene flow over time at a landscape level is very limited for outcrossing grasses (Snow 2002; Tolstrup *et al*. 2003). Therefore, we conducted a 4 year survey to evaluate gene flow from the GRCB fields to test our hypothesis that the preventive measurements were not enough to contain the transgene. The objectives of our survey were to determine the presence and distribution of creeping bentgrass and its compatible relatives carrying the *CP4 EPSPS* transgene in the area, as a measure of the establishment and introgression of the transgene in the feral, non-GE populations.

MATERIALS AND METHODS

Area and Distance Surveyed

A 4 year study to assess gene flow from the 162 ha GRCB fields planted in the control area was conducted during late spring and summer starting in 2003. Banks of irrigation canals, ditches and ponds, roadsides and pipeline sides, which were potential habitats for creeping bentgrass and its compatible relatives, were walked to scout for plants. Production fields were not surveyed. In 2003, a total of approximately 80 km of irrigation canal, ditch and pond banks, roadsides and pipelines were surveyed, mainly in the area 300 m around the GRCB production fields. In 2004, the area surveyed was expanded within the control area in general, as well as outside the control area to the NE, to study the consequences of a documented wind event. The total distance scouted in 2004 was approximately 130 km. The two following years, the same general procedure was followed. Even though some sites were revisited, the area surveyed was extended up to a 5 km radius outside the control area. The distance walked in 2005 and 2006 was approximately 200 km each year.

Plant Identification

Correct identification of *Agrostis* species based on morphological characteristics is difficult (Warnke 2003). Therefore, the main effort of the surveys was concentrated from mid July until the end of September, when visible panicles

aided in identification of the species. Also, panicles with seeds could be collected to further determine the incidence of gene flow via pollen.

Glyphosate-Resistant Plant Detection

Gene flow was determined by the occurrence of individuals of creeping bentgrass and its compatible relatives that contained the *CP4 EPSPS* transgene. Plants found were tested *in situ* for the presence of the CP4 EPSPS protein using Trait✓® RUR strips (Strategic Diagnostics, Newark, DE, USA). A leaf sample of approximately 2-7 mg, generally a 1-2 cm segment, was placed in a 1.5 or 2 mL centrifuge tube with approximately 0.5 mL of distilled water. The tissue was homogenized using a disposable plastic stirrer, then a Trait✓® RUR strip was placed in the tube. After a maximum of 5 minutes, plants were categorized as GR or susceptible. The reliability of the Trait✓® RUR strips to detect the CP4 EPSPS protein was previously determined with PCR and sequencing techniques and found to be 100% accurate in GRCB (Watrud *et al.* 2004). At sites with many plants present, tissue from up to 10 plants was pooled and tested in a single tube. If the test was positive for resistance, then 40% of the plants were tested individually and if all proved to be GR the rest of the plants in the patch were considered “putative” GR, even though not all plants were tested.

Map Construction

Plants were geo-referenced using a hand-held eTrex Legend™ global positioning system (GPS) (Garmin, Olathe, KS, USA). When patches of several

creeping bentgrass or redtop plants were found, plants were counted to the extent that it was possible to identify individual plants, but only one GPS location was recorded. Therefore, a single map point may represent more than one plant. When it was impossible to determine if a patch was a large single plant or several smaller plants, the patch was recorded as a single plant. Maps were prepared using ESRI® ArcMap™ 9.1 (ESRI Inc., Redlands, CA, USA).

RESULTS

In the 2003 *in situ* survey, none of the 57 plants tested near the GRCB production fields carried the *CP4 EPSPS* transgene. Within those 57 plants, 14% were feral creeping bentgrass, 84% were redtop and 2% were rabbitfoot grass (Table 2-1). However, 0.376% of the seeds from creeping bentgrass and redtop panicles collected in 2003 resulted in GR seedlings when tested in the greenhouse (Mallory-Smith, Butler & Campbell 2005) (Fig. 2-1).

In 2004, GR plants were found along canals and irrigation ditches throughout the control area. Creeping bentgrass plants were found in locations where they were not present in 2003 (Fig. 2-2). A total of 300 plants were tested *in situ* in 2004, 49% of which were identified as creeping bentgrass. Redtop and rabbitfoot grass accounted for 37 and 10% of the total plants tested, respectively (Table 1). Due to the inability to identify some plants to species *in situ*, the remaining 4% of the plants were identified as *Agrostis* spp. or potential hybrids with creeping bentgrass. Although, overall, 46% of the plants tested carried the *CP4 EPSPS* gene, 93% of the 148 tested creeping bentgrass plants were GR (Fig. 2) and a further 270 plants were designated as putative GRCB (increasing the possible percentage to 97.5% GR). None of the redtop, rabbitfoot grass, other *Agrostis* spp. or potential interspecific hybrid plants tested was GR. The most distant GRCB plant found was 1.9 km from the closest original GRCB production field. Based on the approximately 130 km surveyed in 2004, the GR plant

Table 2-1. Number of glyphosate-susceptible (S), glyphosate-resistant (R), and putative glyphosate-resistant (P) plants for each species found *in situ* in 2003, 2004, 2005, and 2006.

	2003*			2004			2005			2006		
Species	S	R	P†	S	R	P	S	R	P	S	R	P
Agrostis stolonifera	8	0	0	10	138	270	447	521	437	225	360	427
Agrostis gigantea	48	0		110	0		249	0		374	0	
Polypogon monspeliensis	1	0		29	0		6	0		45	0	
Agrostis spp.	0	0		10	0		65	0		61	0	
Potential hybrid	0	0		3	0		1	1		3	5	
Total	57	0	0	162	138	270	768	522	437	708	365	427

* Due to limitations of the survey, it is not appropriate to compare between years.
† Putative glyphosate-resistant: plant that was within a group of glyphosate-resistant plants but was not tested.

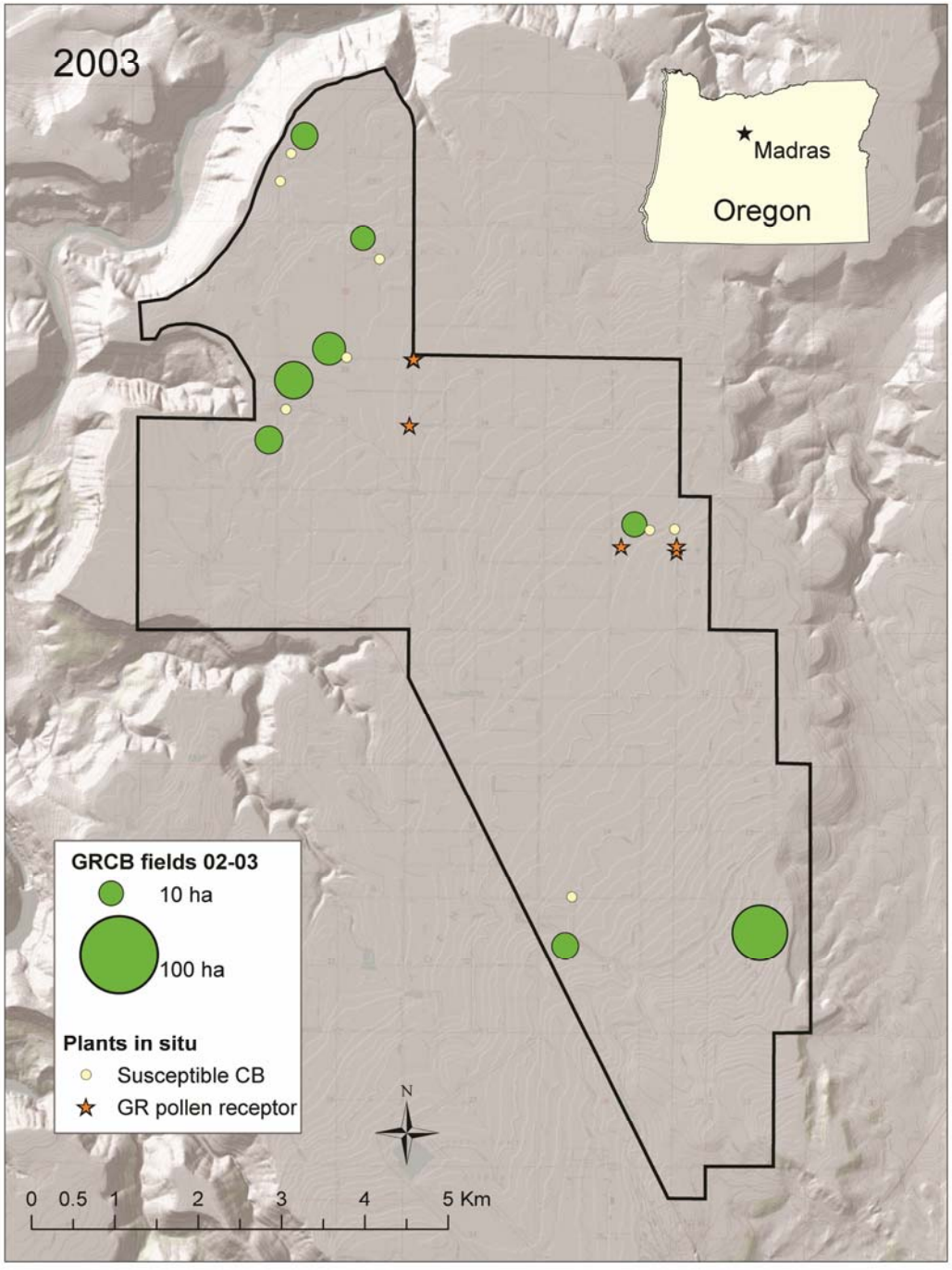

Figure 2-1. Creeping bentgrass (CB) plants tested *in situ* in 2003. Location of transgenic glyphosate-resistant CB (GRCB) fields in 2003 (fields to scale, but not true to shape), susceptible CB plants found *in situ* (a single point may represent more than one plant), and susceptible plants that produced glyphosate-resistant seedlings in 2003 (GR pollen receptor). The black line is the limit of the GRCB control area. Inserted map shows the location of Madras in relation to the state of Oregon.

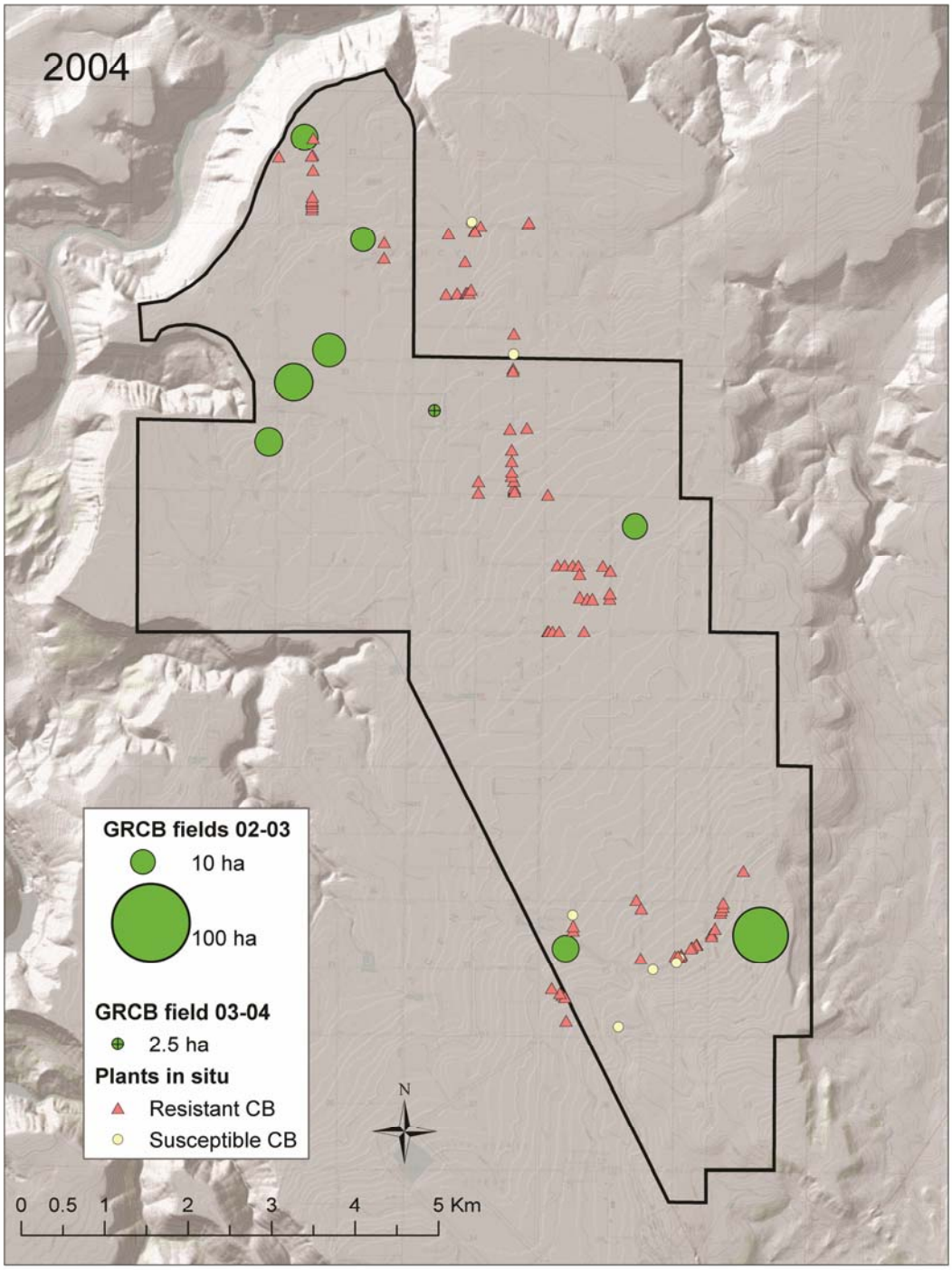

Figure 2-2. Creeping bentgrass (CB) plants tested *in situ* in 2004. Location of transgenic glyphosate-resistant CB (GRCB) fields in 2003 and 2004 (fields to scale, but not true to shape), and glyphosate-resistant and susceptible CB plants found *in situ* (a single point may represent more than one plant). The black line is the limit of the GRCB control area.

abundance was approximately 1.1 plants km^{-1}(3.1 plants km^{-1} if putative GRCB plants are considered).

In 2005, a total of 1290 plants were tested *in situ*: 75% were identified as creeping bentgrass, 19.3% as redtop, 0.5% as rabbitfoot grass, and the remaining 5.2% was represented by *Agrostis* spp. and potential hybrids (Table 2-1). Overall, 40.5% of the plants tested were GR. The GR plants were identified as creeping bentgrass, except for one potential interspecific hybrid. Of the 968 creeping bentgrass plants tested, 54% were GR (Fig. 2-3) or 68% if the 437 putative GRCB plants are considered. Creeping bentgrass plants were generally found close to or in the water, while redtop plants were generally found higher on the banks. The most distant GRCB plant found was 4.6 km from the closest original GRCB production field. The approximately 200 km surveyed resulted in a GR plant abundance of approximately 2.6 plants km^{-1} for 2005 (4.8 plants km^{-1}including putative GRCB plants).

In 2006, sites that were heavily infested with GRCB in 2005 were revisited to assess the persistence of GRCB. In addition, the survey area was extended to more distant areas outside the control area. A total of 1073 plants were tested for glyphosate resistance *in situ*. Of the 1073 plants tested, 55% were creeping bentgrass, 35% were redtop, 4% were rabbitfoot grass, and the remaining 6% were classified as *Agrostis* spp. or potential hybrids (Table 2-1). Overall, 34% of the total plants tested were GR. The GR plants were identified as creeping bentgrass except for five potential interspecific hybrids. Of the 585 creeping bentgrass plants tested, 62% were GR (Fig. 2-4) or 78% if the 427putative GRCB

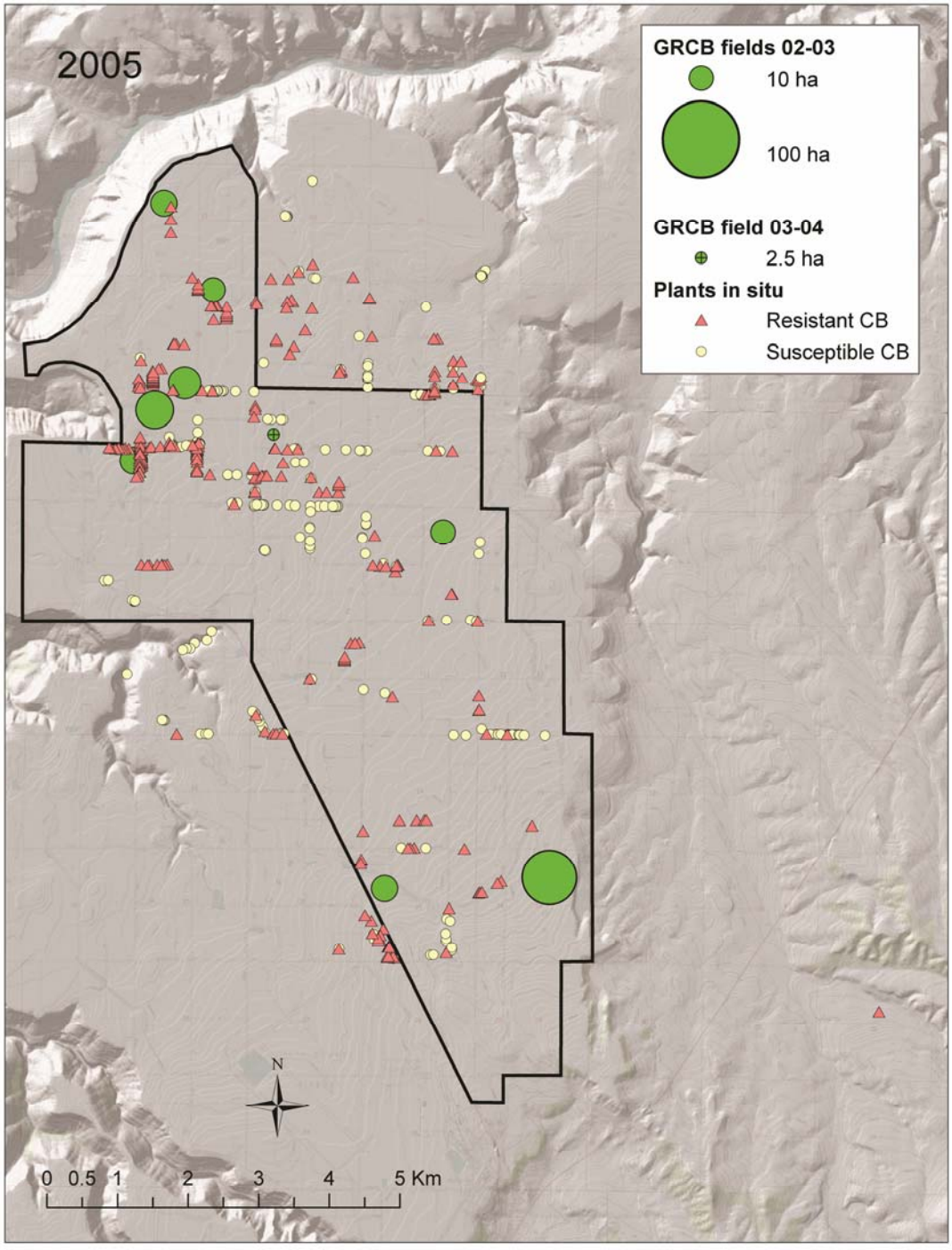

Figure 2-3. Creeping bentgrass (CB) plants tested *in situ* in 2005. Location of transgenic glyphosate-resistant CB (GRCB) fields in 2003 and 2004 (fields to scale, but not true to shape), and glyphosate-resistant and susceptible CB plants found *in situ* (a single point may represent more than one plant). The black line is the limit of the GRCB control area.

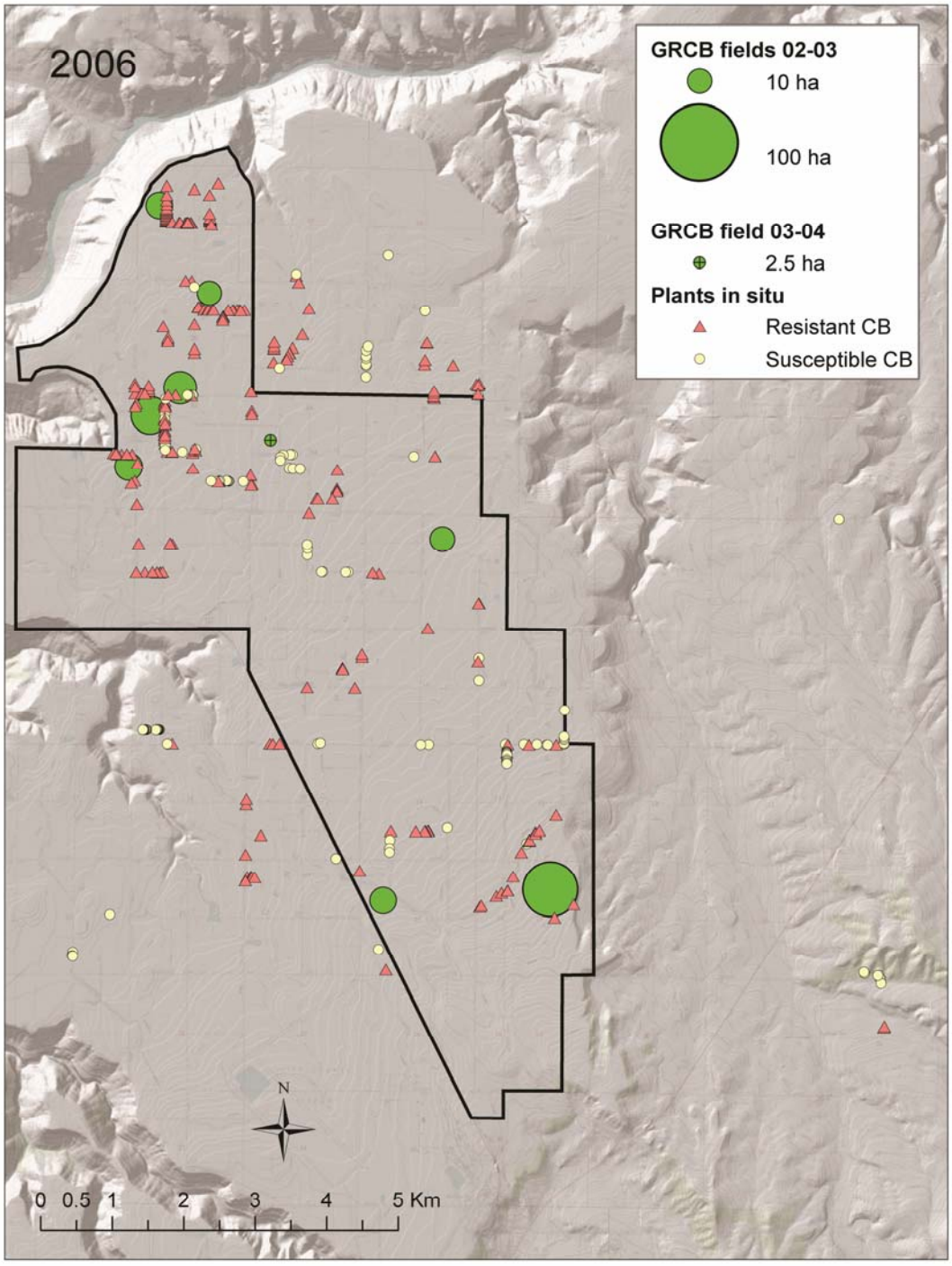

Figure 2-4. Creeping bentgrass (CB) plants tested *in situ* in 2006. Location of transgenic glyphosate-resistant CB (GRCB) fields in 2003 and 2004 (fields to scale, but not true to shape), and glyphosate-resistant and susceptible CB plants found *in situ* (a single point may represent more than one plant). The black line is the limit of the GRCB control area.

were included. In 2006, the most distant GRCB plant was 4.6 km away from an original GRCB field. For, the approximately 200 km surveyed in 2006, the GR plant abundance was approximately 1.8 plants km^{-1}, and 4.0 GR plants km^{-1} if the putative GRCB plants are considered.

It is important to note that the area surveyed in 2005 and 2006 was considerably larger than that of 2004, which was in turn larger than the area surveyed in 2003. Even though the distances walked in 2005 and 2006 were similar, and some previous sites were revisited, not all the sites were the same. No direct comparison of total number of plants or plant density can be performed among years. Because of the regulated status of GRCB, USDA-APHIS required that The Scotts Company remove GRCB plants in the area each year, so we were unable to follow all plants over time. However, some sites had GR plants that persisted throughout the three years.

DISCUSSION

The introduction of GRCB to the control area was a unique and novel starting point from which to evaluate transgene flow from a regulated GE crop to its relatives at a landscape level. The results show that the *CP4 EPSPS* transgene escaped from the GRCB fields and continued to spread for three years after the fields were taken out of production. As we hypothesized, it was unrealistic to think that a transgene could be contained in an outcrossing, wind-pollinated, small seeded, perennial crop, even with expanded isolation distances and stringent production practices. This fact has implications for the deregulation and production of GE crops in the future, especially those for pharmaceutical or industrial uses. It should be noted that although GRCB was introduced in the area in 2001, less than 1 ha was planted and seed was hand harvested in 2002 (The Scotts Company, personal communication) and no GRCB plants were found *in situ* in 2003. We therefore are confident that the main *CP4 EPSPS* transgene source was the 162 ha planted to GRCB in 2002.

Although no plants were found *in situ* expressing the *CP4 EPSPS* gene in 2003, pollen-mediated transgene flow was found in the progeny of 2003 plants (Mallory-Smith *et al.* 2005). These findings are in accordance with Watrud *et al.* (2004) who reported *CP4 EPSPS* gene flow via pollen to susceptible sentinel and resident creeping bentgrass plants, as well as to resident redtop plants, within a year, mostly within 2 km of the control area perimeter and in the direction of prevailing NW winds.

In August 2003, there was a documented strong NW wind event in the production control area that moved seed and panicles from swathed windrows of the northernmost GRCB production field (local growers & The Scotts Company, personal communication). This wind storm is considered to be responsible for the presence of GR plants found SE of that field, in places where no creeping bentgrass plants were identified before (Fig. 2-4), implying gene flow via seed. Previous studies have reported the importance of wind events in determining the directionality of gene flow via pollen but not via seed (Giddings, Sackville Hamilton & Hayward 1997; Watrud *et al.* 2004; Halsey *et al*. 2005; Hoyle & Cresswell 2007). Because creeping bentgrass can easily propagate vegetatively (Carrier 1924), stolons also have to be regarded as a potential mechanism of gene flow in creeping bentgrass (Dysart & Mallory-Smith 2006), with waterways likely to be an important route for both GRCB seed and stolon dispersal.

The means by which the *CP4 EPSPS* transgene moved (pollen, seed, or vegetative propagules) is difficult to determine. Analysis of nuclear ribosomal ITS1-5.8S-ITS2 (ITS) and maternally inherited chloroplast *trnK* intron maturase (*matK*) gene trees of the nine GR plants found by Reichman *et al.* (2006) suggested that establishment resulted from both pollen-mediated intraspecific hybridizations and seed dispersal. Current research aims to clarify how the transgene moved in the hundreds of GR plants we found *in situ* during the four years of our survey.

The 46% of GR plants we found *in situ* in 2004 is orders of magnitude greater than the 0.04% in the 2004-2005 samples tested by Reichman *et al.*

(2006). The difference could be due to the fact that Reichman *et al.* (2006) surveyed exclusively in a 4.8 km zone beyond the control area, while our survey was conducted mainly inside the control area, and outside the control area downwind from the field where the reported wind event took place (Fig. 2). In addition, methodologies differed between the studies. Reichman *et al.* (2006) pooled between 40-50 leaf segments, each representing one plant, while we pooled a maximum of 10 leaf segments in a sample.

The fact that 62% of creeping bentgrass plants tested in 2006 were GR, even after an intense and extended mitigation program was initiated and is still underway, supports Marvier & Van Acker's (2005) opinion that elimination of escaped transgenes is unlikely to be feasible. It will be particularly difficult in cases like GRCB (Fei & Nelson 2004; Carter *et al.* 2005) where the transgene does not have a fitness cost (Ellstrand 2003b; Andow & Zwahlen 2006).

Unlike traits like drought or salt tolerance and disease resistance, which could increase the fitness of the transgenic plant and result in an even greater frequency of gene flow and introgression, GRCB plants do not have a competitive advantage over non-GE plants unless glyphosate is applied (Belanger *et al.* 2003a; Gardner, Danneberger & Nelson 2004). Glyphosate was sprayed on some of the canal and ditch banks in the area, which could have favoured the establishment and persistence of GRCB. However, we think that the high percentage of plants found in 2004 was due mainly to seed dispersion from the production fields during 2003.

In perennial species, such as creeping bentgrass, one plant can contribute its transgene to the population for more than one year, increasing the possibility of introgression with feral creeping bentgrass and compatible native and naturalized species. Under standard agronomic production scenarios, creeping bentgrass is often maintained in production for up to 5 years. Therefore, the standard practices would further increase the likelihood and frequency of transgene escape. Although we found GRCB at 4.6 km from the closest original GRCB field, it is not possible to know or predict how far the transgene has moved. Any extrapolation beyond the most distant point tested would be inappropriate (Ellstrand 2003a). In addition, because there were eight GRCB fields, it is not possible to identify which one was the transgene source for any of the GR plants found. It is advisable that if any other planting of a regulated GE event is authorized, it should be planted at a single site to facilitate the estimation of gene flow and of required isolation distances.

Agrostis spp. plants are plastic and variable in morphology, which makes it difficult to identify hybrids. Although we have not yet confirmed the presence of any interspecific hybrids *in situ*, GR plants originating from seeds collected from susceptible redtop plants have been identified in greenhouse screenings (Zapiola *et al.* 2007). Whether F_1 hybrids produce viable pollen and seed is unknown, but prior studies suggest that intra- and interspecific hybrids can have high fertility (Wipff 2002). Because we found plants with intermediate morphology, we believe it is only a matter of time until we confirm the first hybrid *in situ*.

Glyphosate-resistant creeping bentgrass is the first GE turfgrass species being considered for deregulation and, even though strict preventive measurements were taken, the transgene escaped and is widespread in plant populations in the area 3 years after the original transgene source was removed. These findings highlight the potential of transgene flow from creeping bentgrass, and question the potential for coexistence of GRCB and non-GE grass seed crops. In our opinion, gene flow from GRCB is a greater challenge for agronomic production than it is an environmental risk. Due to the inherent differences in species biology and the traits conferred by transgenes, it is impossible to generalize the potential of transgene flow and persistence for all GE crops. However, the information presented here should be considered when evaluating the use of genetic engineering technology in outcrossing, wind-pollinated, perennial, small seeded crops, and when designing risk assessments for the of release and deregulation of such GE crops. High diligence is required for traits that confer enhanced fitness or that may represent a threat to the environment.

ACKNOWLEDGEMENTS

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CHAPTER 3: FOUR-YEAR ASSESSMENT OF POLLEN-MEDIATED GENE FLOW FROM TRANSGENIC CREEPING BENTGRASS (*Agrostis stolonifera* L.) AT THE LANDSCAPE LEVEL

ABSTRACT

The introduction of transgenic glyphosate-resistant (GR) creeping bentgrass in the environment in 2002 presented a unique opportunity to study gene flow from an outcrossing species at the landscape level. While conducting an *in situ* survey of resistant and susceptible plants, panicles were collected from *Agrostis* spp. and rabbitfoot grass plants for four years. Seeds from the panicles were planted, and seedlings were screened in the greenhouse for expression of the glyphosate resistance transgene. Because some pollen receptor plants could not be positively identified in the field, a *mat*K indel and chloroplast microsatellite (cpSSR) markers in combination with nuclear ITS sequence analysis were used to aid in identification. Transgenic interspecific and intergeneric hybrids seedlings found in the screening also were confirmed using the chloroplast molecular markers and the ITS sequence. Gene flow via pollen was found in all four years. Interspecific hybrids were produced *in situ* three of the four years and one intergeneric hybrid was produced *in situ* in 2005.

INTRODUCTION

Genetically engineered (GE) crops in the USA must go through a deregulation process that focuses primarily on the characteristics of the event before they can be commercially sold (Nap et al. 2003). Gene flow from transgenic crops to feral populations and naturalized compatible relatives has been raised as one of the main issues for the deregulation of transgenic events (Daniell 2002; Messeguer 2003; Marvier & Van Acker 2005; Mallory-Smith & Zapiola 2008).

Creeping bentgrass (*Agrostis stolonifera* L.) is a perennial, outcrossing, wind pollinated, tetraploid, cosmopolitan species that propagates through seeds and stolons (Warnke 2003). Creeping bentgrass is of economic importance because it is a high quality cool-season turf grass primarily used on golf course tees, greens and fairways (Turgeon 1996). Grass weeds in golf courses are a critical problem because they affect the aesthetic appearance and quality of the playing surface, and are difficult and expensive to control (Gange et al. 1999; Belanger et al. 2003). Glyphosate is a broad spectrum, nonselective, systemic herbicide. Therefore transgenic glyphosate-resistant (GR) creeping bentgrass (GRCB) could simplify weed control, of both grasses and broadleaves, on golf courses.

Transgenic GRCB (Roundup Ready®) was developed but is still under U.S. Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS) regulated status. In 2002, while being a regulated article, 162 ha

of GRCB were planted within a 4,500 ha seed production control area north of Madras (44° 38’ 1” N, 121° 7’ 42” W), Oregon, USA, established by the Oregon Department of Agriculture. The transgenic GRCB fields produced pollen and seed for one year. A strong wind event that moved panicles with seeds off the production fields was documented and the GRCB fields were removed after harvest in summer 2003. However, a 2.4 ha transgenic field was planted in 2003 and produced seed in 2004.

Creeping bentgrass and other *Agrostis* spp., including redtop (*A. gigantea* Roth), form a complex of interpollinating, cross-compatible species. The species in the complex produce interspecific hybrids with varying degrees of pollen fertility and seed set (Wipff & Fricker 2001; Belanger et al. 2003; Armstrong et al. 2005). In addition to *Agrostis* species being extremely variable, interspecific hybridization contributes to the difficulty of species identification (Edgar & Forde 1991). Creeping bentgrass also has been reported to form an intergeneric hybrid with rabbitfoot grass (*Polypogon monspeliensis* (L.) Desfontaines) (Wipff & Fricker 2001) which is called perennial beard-grass (x *Agropogon littoralis* (Sm.) Hubbard) (Hubbard 1992). Both redtop and rabbitfoot grass were present in the region where the GRCB seed production control area was established.

The introduction of transgenic GRCB in the environment presented a unique opportunity to study gene flow from an outcrossing species at the landscape level. Seed production practices, including isolation distances, within the control area were strictly regulated in an attempt to prevent transgene flow to non-GRCB fields and to compatible species (Oregon Administrative Rule 603-

052-1240). The Scotts Company further increased the distance from the GRCB fields to be kept free of Agrostis spp. to 300 m. When analyzing the risks of gene flow after the introduction of transgenic crops, it is important to determine if the transgene will spread from the crop plants, if the transgenic crop will hybridize with resident compatible relatives, and how critical of a problem the transgene would be when it escapes (Rieger et al. 1999).

The objective of this research was to assess, during four years, the occurrence of gene flow via pollen at the landscape level to confirm the introgression of the transgene in feral populations of creeping bentgrass and related species. Because definitive identification of *Agrostis* species based solely on morphological characteristics is difficult, and even more complicated when potential interspecific and intergeneric hybrids are involved, we used a *mat*K insertion-deletion (indel) marker and chloroplast microsatellites (cpSSR) markers in combination with nuclear ribosomal DNA internal transcribed spacer (ITS) sequences to aid in the confirmation of the putative hybrids found *in situ* and in the seedling screening.

MATERIALS AND METHODS

Plant Material

From 2003 through 2006, panicles from creeping bentgrass, redtop, rabbitfoot grass, and *Agrostis* spp. were collected from within the GRCB seed production control area and up to 5 km outside the control area. Panicles were hand-threshed individually. Seeds were planted in nine rows in 27.5 x 52.5 x 5 cm trays using potting mix and grown in the greenhouse. Total genomic DNA was extracted from dried young leaves of plants tested *in situ* and from fresh young leaves of seedlings grown in the greenhouse using the DNeasy 96 Plant kit (Qiagen, Valencia, CA) and DNeasy Plant Mini Kit (Qiagen).

Herbicide Screening

Glyphosate resistance was confirmed at three levels: phenotype, protein expression, and genotype. Two weeks after planting, when most seedlings were at the two-leaf stage, seedlings were counted and sprayed with glyphosate at 1.68 kg a.e. ha^{-1}. Fourteen days after application, surviving seedlings were counted and resprayed with the same rate of glyphosate. Twenty-one days after the second application, a final count was performed and surviving seedlings were tested with Trait✓® RUR lateral flow strips (Strategic Diagnostics, Newark, DE, USA) to confirm the expression of the glyphosate-resistant 5-enolpyruvyl-shikimate-3-phosphate synthase protein from *Agrobacterium* sp. strain CP4 (CP4EPSPS) encoded by the glyphosate resistance transgene.

A subset of plants was further evaluated to confirm the presence of the *CP4EPSPS* transgene by PCR. We designed and used primers CP4F ACGATTTCGACAGCACCTTC and CP4R TGCAGCATCTTTTCCGTATG. The PCR mixture (15 µL) contained 2-5 ng of genomic DNA, 1x CoralLoad buffer, 1 x Q-solution, 200 µM of each dNTP, 0.5 µM of each primer, and 0.5 U reaction^{-1} *Taq* DNA Polymerase (Qiagen). The PCR program consisted of: 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 20 s at 55 °C, and 30 s at 72 °C, with a final extension of 10 min at 72 °C, using a C1000TM Thermal Cycler (Bio-Rad, Hercules, CA). Presence of PCR amplicons was confirmed by UV fluorescence after electrophoresis on 2% agarose gels stained with ethidium bromide.

Hybrid Confirmation

cpSSR. The 12 *Agrostis* cpSSRs markers developed (see Chapter 5 for primer and amplification conditions) were used to identify the chloroplast type.

matK. Based on alignments of *mat*K sequences (GeneBank accessions DQ146797-DQ146826) from velvet bentgrass (*A. canina L*.), colonial bentgrass (*A. capillaris* L.), dryland bentgrass (*A. castellana* Boiss. & Reuter), spike bentgrass (*A. exarata* Trin.), redtop, Idaho bentgrass (*A. idahoensis* Nash), northern bentgrass (*A. mertensii* Trin.), seashore bentgrass (*A. pallens* Trin.), rough bentgrass (*A. scabra* Willd.), creeping bentgrass, brown bentgrass (*A. vinealis* Schreber), rabbitfoot grass, and water bent (*P. viridis* (Gouan) Breistr.), a new pair of primers was designed to amplify a segment that contains a 18bp duplication for some, but not all of the species. Primers pairs were designed using

Primer3 (Rozen & Skaletsky 2000). The PCR mixture (15 µL) contained 2-5 ng of genomic DNA, 1x CoralLoad buffer, 200 µM each dNTP, 0.5 µM each primer, and 0.5 U reaction^{-1} *Taq* DNA Polymerase (Qiagen). The PCR program consisted of: 2 min at 94 °C, followed by 30 cycles of 20 s at 94 °C, 20 s at 56 °C, and 35 s at 72 °C, with a final extension of 10 min at 72 °C, using a C1000TM Thermal Cycler (Bio-Rad). PCR amplification products were visualized by UV fluorescence after electrophoresis on 4% agarose gels stained with ethidium bromide.

ITS. Primers used to amplify the nuclear ribosomal ITS1-5.8S-ITS2 (ITS) sequence were ITS5 GGAAGTAAAAGTCGTAACAAGG (White et al. 1990) and P216R CGTCGTGCGCACCGTTCAWAGGG (Reichman et al. 2006) in a 25 µL PCR mixture, following a modification of the Reichman et al. (2006) protocol, using 30 s at 56 °C for annealing. PCR amplicons were cloned with TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Eight to 10 clones per plant were screened by PCR. PCR products were purified using QIAquick PCR Purification Kits (Qiagen), and sequenced at the CGRB Core Laboratories at Oregon State University on an ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Carlsbad, CA).

Analysis. Plants were separated in two groups based on their matK indel marker amplification size (124 for creeping bentgrass, and 106 for redtop and rabbitfoot grass) and cpSSR data were analyzed in Structurama (Huelsenbeck & Andolfato 2007) using a model with expected number of populations as a random variable and Markov chain Monte Carlo of 10,000 cycles to determine the number

of subpopulations (K). A set of 23 known creeping bentgrass, 16 redtop and 12 rabbitfoot grass plants was used as a reference.

Nuclear ITS sequences were aligned in S CLC Sequence Viewer 5.0 (Knudsen et al. www.cicbio.com) for each plant and a consensus sequence was generated. In the cases where two distinct types of sequences were observed, implying hybridization, a consensus sequence for each type was generated. The UPGMA tree was created in S CLC Sequence Viewer 5.0 using the ITS sequences (GeneBank accessions DQ146766-DQ146796) from velvet bentgrass, colonial bentgrass, dryland bentgrass, spike bentgrass, redtop, Idaho bentgrass, northern bentgrass, creeping bentgrass, rough bentgrass, and rabbitfoot grass, as references.

In cases where we had plants identified as *Agrostis* spp. or we suspected that hybrid plants could be pollen receptors, ITS sequences of the mother plants, when tissue sample was available, or otherwise from the seedlings, were compared with chloroplast information to confirm species, interspecific, and intergeneric hybrids, based on placement of the plants in the chloroplast type group versus the nuclear ITS tree. The same method was used to confirm the putative hybrids among the GR seedlings detected.

RESULTS

Evidence of gene flow via pollen was found in all four years. Of the 15,963 seedlings tested from seed originating from susceptible plants in 2003, 0.363% were GR. The percentage of resistant seedlings from seed originating on susceptible plants was 0.032% of 206,971, 0.036% of 44,257 and 0.006% of 104,832 seedlings screened for 2004, 2005 and 2006, respectively (Table 3-1).

While we did not find any GR seedling originating from rabbitfoot grass plants, we did find GR seedlings originating from redtop plants in three of the four years and GR seedlings originating from other *Agrostis* spp. or potential hybrids with creeping bentgrass in 2004 and 2006 (Table 3-1).

The GR seedlings resulted from panicles collected from 7, 6, 4, and 3 susceptible plants in 2003, 2004, 2005, and 2006, respectively (Table 3-2). While the percentage of *in situ* sampled plants that produced GR seedlings averaged 28% for 2003, it was 6.12, 2.80 and 1.75% for 2004, 2005 and 2006, respectively. The proportion of creeping bentgrass susceptible plants that yielded GR seedlings was greater than that of redtop plants except for 2006. Because the area sampled was not the same for each year, direct comparison of the results from different years is not possible. The locations of the glyphosate-susceptible plants that produced GR seedlings are presented in Fig. 3-1.

The percentage of resistant seedlings from seed originated from established GRCB plants was 99% of 451, 90% of 3591, and 94% of 484

Table 3-1. Number of glyphosate-susceptible (S) and glyphosate-resistant (R) seedlings of seed collected *in situ* from susceptible plants of each species from 2003 through 2006.

	2003		2004		2005		2006	
Species	S	R	S	R	S	R	S	R
Rabbitfoot grass	528	0	62,999	0	13,847	0	45,852	0
Redtop	14,322	29	138,909	51	21,585	0	51,275	2
Creeping bentgrass	1,034	29	710	9	6,378	16	3,494	0
Agrostis spp.	21	0	4,287	6	2,431	0	4,205	4
Total	15,905	58	206,905	66	44,241	16	104,826	6

Table 3-2. Number of susceptible plants that produced transgenic glyphosate-resistant seedlings (R) of the total number of glyphosate-susceptible plants (Plants) of each species from which panicles were collected and seed tested for 2003 through 2006. The % R is the percentage of susceptible plants sampled that produced glyphosate-resistant seedlings.

	2003			2004			2005			2006		
Species	Plants	R	% R	Plants	R	% R	Plants	R	% R	Plants	R	% R
Rabbitfoot grass	1	0	0	11	0	0.00	5	0	0	22	0	0.00
Redtop	17	4	23.5	72	1	1.4	67	0	0	107	2	1.87
Creeping bentgrass	4	3	75	5	3	60	53	4	7.6	27	0	0
Agrostis spp.	3	0	0	10	2	20	18	0	0	15	1	6.7
Total	25	7	28	98	6	6.1	143	4	2.8	171	3	1.8

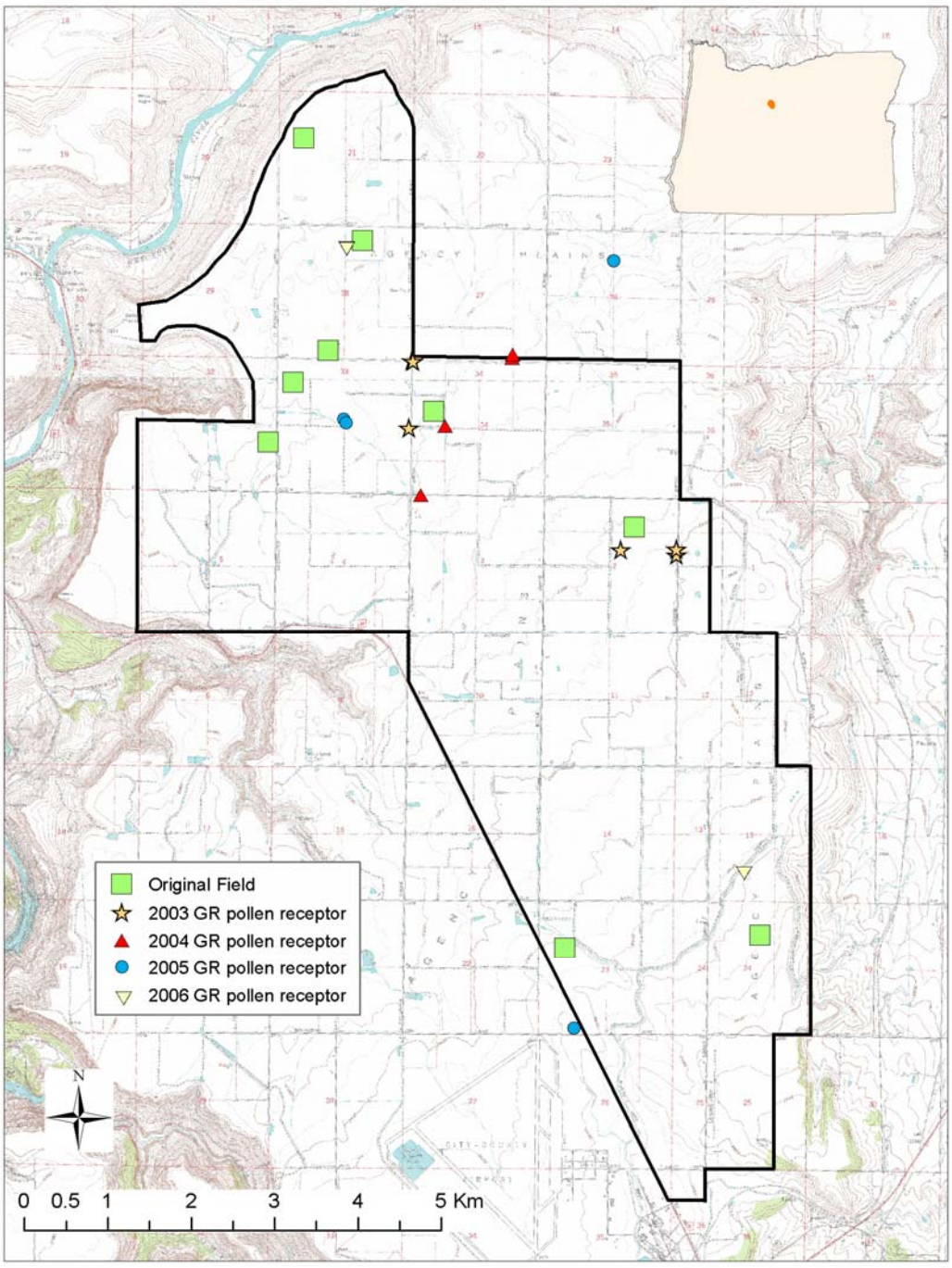

Figure 3-1. Location of susceptible plants that produced transgenic glyphosate-resistant (GR) seedlings (GR pollen receptor) in the four years. A single point may represent more than one plant. The black line is the limit of the GRCB seed production control area. The inserted map shows the location of the control area in relation to the state of Oregon, USA.

seedlings screened for 2004, 2005 and 2006, respectively. The number of GRCB plants from which panicles were collected was 10, 56, and 14 for 2004, 2005 and 2006, respectively. While all the plants produced at least one GR seedling in 2004, there were 3 plants, in each 2005 and 2006, that only had one seedling screened and they were susceptible.

The cpSSR markers revealed a great variability within species (Chapter 5). Chloroplast information alone was enough to confirm the formation of redtop x GRCB hybrids. The hybrid seedlings originating on susceptible redtop plants were confirmed by *matK* indel and cpSSR markers and by ITS nuclear sequences (Table 3-3; Fig. 3-2). One of the eight seedlings, all GR, produced on a single GRCB had an unusual phenotype. Further studies using the combination of cpSSR markers and ITS sequences revealed that it was a GRCB x rabbitfoot grass intergeneric hybrid.

Some mother plants were identified only to *Agrostis* spp. or were suspected of being interspecific hybrids. The use of *matK* indel and cpSSR markers and ITS sequences in combination with morphology, mainly the presence/absence of stolons and rhizomes, were useful to identify most of the plants (Table 3-3). However, there are still some plants where the information gathered is not enough and prevent us from concluding whether they are hybrids or not .

Table 3-3. Genetic characteristics of mother plants tested *in situ* and seedlings screened. Type of mother plant (Mpl), GR trait of mother plant (GRMpl), type of seedling (Type), GR trait of plant (GR), matK indel allele (matK), chloroplast type (cpSSR), ITS sequence type (ITS), additional observations, and species/hybrid call based on all information.

Plant ID	Year	Mpl†	GRMpl‡	Type	GR	matK	cpSSR	ITS	Observations	Call
Mplants										
15	2003	-	-	CB	S	124	CB1	CB		CB x CB
090	2005	-	-	CB?	GR	124	CB1	CB	shaded upright growth	CB x CB
405	2006	-	-	RT x CB?	S	124	CB2	CB	3/4sdlg from plant w/ rhizomes	CB x CB?
483	2006	-	-	RT	S	106	RT2	RT		RT x RT
M008A	2005	-	-	Hyb?	S	124	CB2	CB	5/5 seedlings w/o rhizomes	CB x CB
M397	2005	-	-	CB	S	124	CB2	CB		CB x CB
M543	2006	-	-	Hyb?	GR	124	CB1	CB		CB x CB
M627	2006	-	-	Hyb?	GR	124	CB1	CB	bunch type, rhizomes?	CB x CB?
M647	2006	-	-	Hyb?	GR	124	CB1	CB	rhizomes or burried stolons	CB x CB
M820	2006	-	-	Hyb?	GR	124	CB1	CB	rhizomes?	CB x CB?
M06084	2006	-	-	Hyb?	S	124	CB2	CB, RT	RT like panicle, stolons	CB x RT
M06102	2006	-	-	RT	S	n/a	n/a	RT		RT x RT
Seedlings										
12-6_2	2004	Hyb?	S	Hyb? x CB	GR	124	CB1	CB	no rhizomes	CB x CB
12-8_2	2004	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	no rhizomes	CB x CB
13-3_1	2004	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	no rhizomes	CB x CB
13-4_3	2004	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	rhizomes	CB x CB?
13-4_4	2004	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	rhizomes	CB x CB?
13-6_1	2004	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	rhizomes	CB x CB?
49-6_2	2003	RT	S	RT x CB?	GR	106	RT3	RT, CB		RT x CB
201_1	2005	CB	GR	CB x RF?	GR	124	CB1	CB, RF		CB x RF
332_1	2005	CB	S	CB x RT?	GR	124	CB2	CB	rhizomes	CB x CB?
404_1	2006	RT	S	RT x CB?	GR	106	RT1	RT, CB		RT x CB
405_3	2006	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	rhizomes	CB x CB?
405_4	2006	Hyb?	S	Hyb? x CB	GR	124	CB2	CB	no rhizomes	CB x CB?

† (-) unknown, Hyb?= hybrid?, RT= redtop, CB= creeping bentgrass, RF= rabbitfoot grass.

‡ (-) unknown, S= glyphosate-susceptible, GR= glyphosate-resistant.

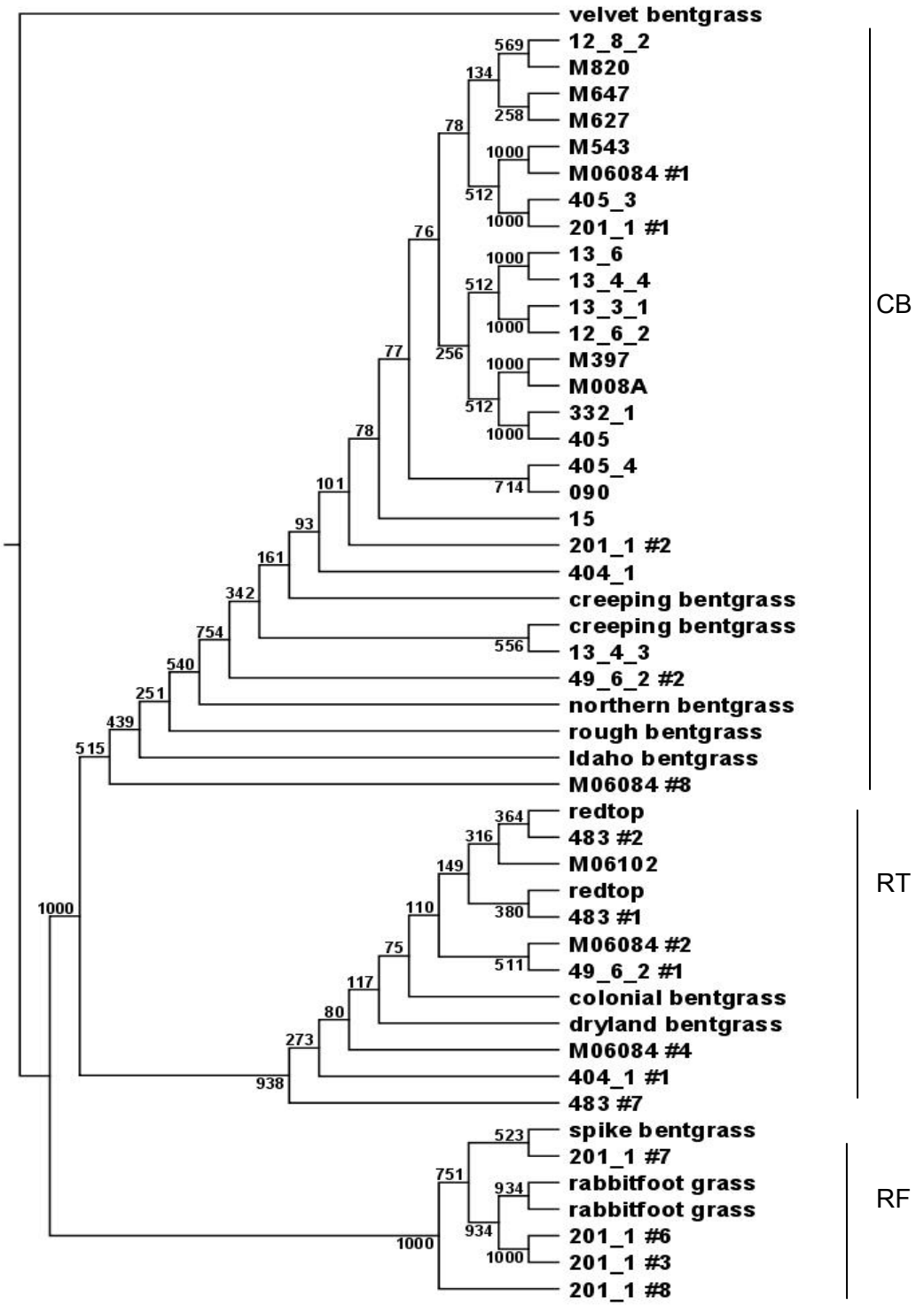

Figure 3-2. Bootstrapped UPGMA tree for ITS sequences. The number following # is the clone number presented when sequences were different. The number following the last underscore implies the plant is a greenhouse screening survival. No underscore means mother plant. Numbers on branches are Boostrap values.

DISCUSSION

Pollen-mediated gene flow is very dependent on species characteristics. Creeping bentgrass has a particularly large potential for pollen-mediated gene flow because it is an outcrossing, wind pollinated, perennial that establishes outside of cultivation and has numerous compatible relatives, with which it forms hybrids, that are sympatric in areas where creeping bentgrass seed is produced. Pollen viability in creeping bentgrass was reported to be 1%, 2 h after release (Fei & Nelson 2003; Pfender et al. 2007).

Other main factors that affect gene flow by pollen are pollen source size, distance from source, pollen receptor size, and environmental conditions. The density of GR plants, and therefore the size of the pollen source, were clearly different in 2003 compared with the other years. However, despite the removal of the 162 ha GRCB seed production fields in 2003, there was still enough pollen carrying the transgene in subsequent years to allow for the persistence and further dissemination of the transgene in the environment. Our results confirm the introgression of the glyphosate-resistance transgene in resident *Agrostis* spp. populations. Feral GR plants established along irrigation canals, ditches, pipes and any other water body were a continuous source of pollen that allowed pollen-mediated gene flow to occur over the four years.

The 2.8 and 0.2% pollen-mediated gene flow we found in 2003 is orders of magnitude higher than the 0.03 and 0.04% hybridization for resident creeping bentgrass and redtop, respectively, reported by Watrud et al. (2004). This

difference could be due to slight differences in methodology, but also to the fact that they studied plants solely outside the control area, while we collected panicles from plants located closer to the pollen source of the GRCB production fields.

There is a large difference between the percentage of gene flow via pollen found in the greenhouse screening of seedlings originated on susceptible mother plants and the 62% of GRCB plants found in 2006 during the *in situ* survey (Zapiola et al. 2008). The level of pollen-mediated gene flow reported here can not account for all GR plants found *in situ*, implying that there are other means of gene flow, either seeds or vegetative propagules, that also play a role in perpetuating the transgene in the environment. These findings highlight the potential for gene flow via pollen, but also question the feasibility of male sterility to contain a transgene, because of the other means that allow the transgene to move.

Glyphosate-resistant creeping bentgrass plants do not have a competitive advantage over non-transgenic plants unless glyphosate is applied (Gardner et al. 2004). The frequency of GR plants established outside of cultivation on the banks of irrigation canals, ditches and ponds during the four years was impacted by the mitigation program conducted by The Scotts Company and the application of glyphosate by growers and the irrigation district staff. The fact that there was a mitigation program in place prevented us from following specific plants over time.

The lack of availability of the original transgenic seed planted or produced in the area greatly limited our ability to use the cpSSR markers and nuclear ITS

sequence to identify the degree of relationship of tested plants to the original transgenic cultivar. Fifteen plants from Backspin, the cultivar that was transformed to produce the GE creeping bentgrass, were included as references, but three chloroplast haplotypes were found for that cultivar, preventing us to make any assumptions of the transgenic crop chloroplast haplotype. Also, the lack of availability of the nuclear genotype of the transgenic cultivar prevented the determination of gene movement via seed versus pollen.

The fact that GR seedlings originating from susceptible plants that were identified and confirmed, based on cpSSR and *mat*K indel markers, as redtop emphasizes the potential of creeping bentgrass to produce interspecific hybrids with sympatric compatible species. The hybrid seedlings were vigorous and long-lived, and they have both stolons and rhizomes with varying degrees of development. By using the chloroplast markers and the ITS sequence, we also were able to confirm an interspecific creeping bentgrass x redtop hybrid *in situ* among our susceptible pollen receptors, that we could not have confirmed based solely on morphological characteristics.

Not only were interspecific hybrids produced *in situ*, but we found, and confirmed by means of the cpSSR markers and the ITS sequence, an intergeneric GRCB x rabbitfoot grass hybrid (perennial beard-grass) originated on a resident GRCB plant. This GR perennial beard-grass plant constitutes the first report of an intergeneric transgenic hybrid of a still regulated event found to be produced *in situ.*

Because of the long history of coexistence of creeping bentgrass, redtop and rabbitfoot grass *in situ*, the limited nature of our survey, and the potential for misidentification, we are confident that there were other interspecific, and potentially intergeneric, hybrids established *in situ* that we did not find or identify during the survey.

CONCLUSION

Three years after the removal of the GRCB production fields, the glyphosate-resistance transgene persisted in the environment. Although not part of the survey, we identified GR plants *in situ* in the summer of 2008. The fact that we collected panicles from susceptible plants that yielded GR seedlings every year means that even after the fields were taken out of production there was enough GR pollen produced to compete with non-GR pollen, and it was enough to pollinate other species. The fact that we found interspecific and intergeneric hybrids produced *in situ* confirms the complexity of the system and highlights the difficulties for mitigation practices because there might be plants with intermediate characteristics that may not be identified and destroyed.

Because pollen-mediated gene flow depends on the size of the pollen source, it is critical to note that these results are a consequence of a single introduction at a large scale, and if transgenic crops were planted for seed production and produced pollen every year, the frequency of pollen donor GR plants will increase significantly, especially when paired with glyphosate as a selection pressure.

This research proved the occurrence, for four years, of pollen-mediated gene flow in creeping bentgrass and related species at the landscape level, and contributes to the understanding of the fate of the CP4EPSPS transgene, in an outcrossing, wind-pollinated, perennial grass. Results of this study should be considered when evaluating the deregulation of transgenic outcrossing, wind-

pollinated, perennial crops, and to analyze the potential for coexistence of transgenic and non-transgenic outcrossing grass seed crops. However, because of the inherent differences in species biology and the traits conferred by transgenes, it is not possible to generalize the potential of transgene flow and effect for all transgenic crops. There should be increased diligence when evaluating the deregulation of traits that confer enhanced fitness or that may represent a threat to the environment.

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CHAPTER 4: DEVELOPMENT OF NOVEL CHLOROPLAST MICROSATELLITE MARKERS TO IDENTIFY SPECIES IN THE *Agrostis* COMPLEX (POACEAE) AND RELATED GENERA.

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ABSTRACT

We needed a reliable way to identify species and confirm potential interspecific and intergeneric hybrids in a landscape level study of gene flow from transgenic glyphosate-resistant *Agrostis stolonifera* (Poaceae) to compatible relatives. We developed 12 new polymorphic chloroplast microsatellite markers to aid in identifying species recipient of transgenic pollen both within the *Agrostis* complex and the related genera *Polypogon*.

Keywords: SSR, primers, creeping bentgrass, bentgrass, grasses, hybridization.

INTRODUCTION

Agrostis is considered one of the most difficult and complicated of the grass genera from a taxonomic perspective (Warnke 2003). Many *Agrostis* species, including *A. stolonifera*, *A. gigantea*, *A. capillaris*, and *A. castellana*, are cross compatible and form interspecific hybrids (Wipff & Fricher 2001). *Agrostis stolonifera* also produces intergeneric hybrids with several *Polypogon* species (Björkman 1960). The great challenge of identifying species and potential hybrids based on morphological characteristics has motivated the development of DNA markers to assist in confirming the identification of plants found *in situ*.

Chloroplast markers are useful for species determination and the study of hybrid evolution in plant taxa and seed dispersal because of the uniparental mode of transmission (Ennos et al. 1999). Powell et al. (1995) reported SSR variability in the chloroplast genome and suggested it may be a source of polymorphic markers for studies in plant population genetics and systematics. Chloroplast microsatellites (cpSSR) are now a high resolution tool for examining cytoplasmic variation in a wide range of species (Provan et al. 2001, 2004; McGrath et al. 2006). Our goal was to develop cpSSR markers that amplify in several *Agrostis* species and *P. monspeliensis* to assess maternal lineage of plants recipient of transgenic pollen, and confirm the formation of transgenic hybrids between *A. stolonifera* and compatible relatives.

MATERIALS AND METHODS

The complete chloroplast genome sequence of *A. stolonifera* (GenBank accession no. NC_008591.1) was screened for the presence of mono- and dinucleotide repeats with a minimum of eight and five repeats, respectively, using *Phobos 3.2.6.* (Christoph Mayer, 2006-2009, http://www.rub.de/spezzoo/cm/cm_phobos.htm). A total of 52 cpSSR loci were identified. Primer pairs homologous to the flanking regions were designed for 32 cpSSR loci using Primer3 (Rozen & Skaletsky 2000). Total genomic DNA was extracted from young leaves using the DNeasy 96 Plant kit (Qiagen). The PCR reaction mixture (15 µL) contained 2-5 ng of genomic DNA, 1x Phusion HF buffer, 200 µM each dNTP, 0.4 µM each primer, and 0.02 U μL^{-1} Phusion^{TM} High-Fidelity DNA Polymerase (Finnzymes). The PCR program consisted of: 2 min at 98 °C, followed by 30 cycles of 10 s at 98 °C, 20 s at 60-63 °C (primer-specific), and 20 s at 72 °C, with a final extension of 10 min at 72 °C, using a C1000TM Thermal Cycler (Bio-Rad). Uniformity of PCR amplification products was confirmed by UV fluorescence after electrophoresis on 2% agarose gel with ethidium bromide.

RESULTS AND DISCUSSION

Screening of 32 primer pairs on *A. stolonifera* and *A. gigantea* revealed that 31 amplified in both species, but five gave multiple bands per plant and were discarded. From the remaining 26 primer pairs, 14 were chosen for fluorescent capillary analysis. The 14 selected labeled primer pairs (Table 4-1) were tested on 139 plants representing nine *Agrostis* species and *Polypogon monspeliensis* (Table 4-2). PCR products were multiplexed in two sets (1:400 – 1:1000 dilution) and were screened by capillary electrophoresis using an ABI Prism® 3100 Genetic Analyzer. Genotypes were scored manually using ABI Prism® Genotyper v. 3.7 NT (Applied Biosystems). All primer pairs amplified a product in all species tested except *A. vinealis* ssp. *trinii* and *P. monspeliensis* (Table 4-2). Twelve of the 14 markers were polymorphic, with between four and 11 alleles detected in the 10 species tested. Alleles were shared across species at several loci, but haplotypes were unique in all species tested (Table 4-2). The Acp18 and Acp30b loci repeat motifs in *A. stolonifera* are comparable to those of the *trnK* intron and *rpoC2/rps2* loci reported for rice, wheat, corn, and other grasses by Provan et al. (2004), which suggests the potential cross utility of the novel markers in additional species beyond those evaluated.

Table 4-1. Characterization of 14 chloroplast microsatellite markers for the grass *Agrostis stolonifera*. Position, amplicon expected size, repeat motif, and chloroplast gene and interspacer region associated are expressed relative to *A. stolonifera* chloroplast sequence (NC_008591.1).

Name	F sequence (5'- 3')	R sequence (5' -3')	Genome position	Amplicon size (bp)	Repeat	T_a (°C)	Gene/intergenic spacer
Acp12	CATAATGCGCCGTTTCTATT	CAAGCAAGATTGGTTGGATT	36917	262	$(A)_7C(A)_{10}$	63	*psaB/rps14*
Acp18	CCGTTCCTTTTCTACCCAAT	TGGGTTTTCCTATGAGTGGA	3903	217	$(A)_{11}G(A)_8$	63	*matK/trnK*
Acp19	TTCCACAATGAAATCCAAAA	ATTGGATGGCAAAGATTTCA	19379	199	$(T)_{11}$	60	*trnC/rpoB*
Acp22	AACAAGTGACTCGAACTGTGTG	TCGTTCTTGATCGGAATCAT	56933	129	$(T)_{11}$	63	*rpl23/psaI*
Acp23	TAGGTTCAGGCGAGGTAGTG	AAATAAAAACCACCCTTCAATG	61542	147	$(A)_{11}$	63	*petA/psbJ*
Acp24	AACTCATCTGATTTCCGGTAGA	GGATTTACGAAAGGGTTGCT	65996	240	$(T)_{11}$	63	*rps18/rpl33*
Acp25b	TCCCTCCACTCGCTCTAAAA	GGGCCTTTTATGGCTGATCT	115028	261	$(AG)_6$	63	*ndhH* gene
Acp26b	CCTTCGTGGAGTCCCTTCTT	GGCCCAAAGGGTTAGCAATA	18312	153	$(A)_{12}$	63	*petN/trnC*
Acp27b	GCATCAGCATGTAGGTTCCA	AACACTTGCCTCCGATTGAC	41408	352	$(T)_{12}$	63	*psaA*/*ycf3*
Acp28b	GGTTTGGTGCTTTAGCAGGT	AGCTAGCCCAGCCTTTTTCT	65192	298	$(A)_{12}$	63	*psaJ/rpl33*
Acp29b	CGTCCGAAGGAATCAATGTT	CTGCTTGGCGTTGCATATTA	21167	396	$(A)_{13}$	63	*rpoB* gene
Acp30b	TCAATGGCCAACTCTCAGTG	TGACCAAAATGAACTCCTGCT	29956	223	$(A)_{13}G(A)_6$	63	*rps2*/*rpoC2*
Acp31	AAGGAAACATAGAGTCATAGCAAAT	CCTTGAGGTCTTTGCAATTC	46646	178	$(A)_{13}$	63	*trnT/trnL*
Acp32	CCCCACGATACAATGAATTT	TCCTCTGGACGCGTATAGTAA	57220	94	$(A)_{13}T(A)_6$	63	*rpl23/psaI*

Table 4-2. Allele sizes (bp) of the 12 newly developed polymorphic* cpSSR markers for 9 *Agrostis* species and *Polypogon monspeliensis.*

Species	N†	Acp12	Acp18	Acp19	Acp22	Acp23	Acp24	Acp26b	Acp27b	Acp28b	Acp30b	Acp31	Acp32	N‡
A. stolonifera	57	258 259	213	195	125 126	144	234 235	148 149	349 350 351	292 293 294	218 219 220	172 173 174	86 87	7
A. gigantea	14	258	213 214	193 195	125 126	142 143 145	233 234 235	145 148 149	348 350	292 293	218 219	172 173 174 193	87 88	7
A. capillaris	16	258	213	191 195	124 125	142	232 233 234 235	145	348	292 293	216	189 209	88	9
A. castellana	8	258	212	193 195	123 124	142	233 234 235	145	348	293 294	216	191	88	5
A. scabra	6	258	214	192	125	143	235	147	350	291	219	173	92	1
A. canina	10	258	214	192	124 125	143	235	148	350	293	218	175	91	2
A. exarata	6	257	212	192 193	125 126 127	142	236 237	147	348	292 293	220 221	172 175 176	90	4
A. vinealis	6	257	215	192	124	142	235	148	349	299	217	171	88	1
A. vinealis ssp. *trinii*	6	256	229	-	-	142	228	-	347	288	216	-	-	1
P. monspeliensis	10	257	213	-	125	143	237	145	348	291	218	168	89	1
Total	139	4	5	4	5	4	7	4	5	6	6	11	7	37

* Acp29b (394 bp) and Acp25b (258 bp) were monomorphic for all the species tested and are, therefore, omitted from the table.

† Number of individually tested plants for each of the species.

‡ Number of unique haplotypes detected per species.

- No amplification.

CONCLUSION

We demonstrated that the 14 cpSSR markers amplify in a wide range of *Agrostis* species and related genera, and that 12 are robust and polymorphic. Although the analysis of hybridization past the first generation requires a combination of chloroplast and nuclear markers (Reichman et al. 2006), the primers developed will be useful to identify maternal lineages that participate in the formation of interspecific and intergeneric F_1 hybrids in the *Agrostis* complex. These novel cpSSR markers also can be used to study inheritance of chloroplast lineages and estimate transgene movement via seeds. The cpSSR markers developed can be helpful to assist identification of native vs. introduced *Agrostis* species, although this would need to be determined on a case by case basis due to the demonstrated interfertility of these species. Finally, these cpSSR markers will be a useful tool in *Agrostis* species breeding programs, with the potential to be applied to other genera as well.

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CHAPTER 5: SOAKING TIME AND WATER TEMPERATURE IMPACT ON CREEPING BENTGRASS SEED GERMINATION

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ABSTRACT

Seed deterioration, and therefore seed germination potential, are highly influenced by relative humidity and temperature. However, limited species-specific information is available about the effect of long term soaking in water on seed germination potential. Knowing the potential fate of a creeping bentgrass seed that falls in an irrigation canal was important for the study of transgene flow at the landscape level. The objectives of this study were to evaluate the effect of soaking time and water temperature on germination of creeping bentgrass seed and to assess how fast a panicle could be moved in an irrigation canal. Panicles of three cultivars of creeping bentgrass were soaked in water for up to 17 wk at two water temperatures, 4 and 20 C, and standard germination tests were performed on the seed. Creeping bentgrass seeds did not lose their germination after 17 wk in water at 20 C and, although reduced, germination was still 46% after 17 wk in water at 4 C. The reduction in germination in seeds from panicles kept in water at 4 C was due to the induction of secondary dormancy, which was overcome by dry seed storage at room temperature. We quantified that a panicle that falls in an irrigation canal can travel downstream at an average rate of 19 m min^{-1} and move seeds that could potentially establish seedlings elsewhere. Therefore, movement of creeping bentgrass seed by water has to be considered as a means of gene flow.

Nomenclature: creeping bentgrass, *Agrostis stolonifera* L.

Key words: seed longevity, gene flow, seed dispersal, seed movement, *Agrostis*

INTRODUCTION

Seeds have the remarkable capacity to survive as viable regenerative organs until the conditions are suitable for the beginning of a new generation. However, seeds cannot retain their viability indefinitely and eventually they deteriorate and die (Copeland and McDonald 2001). Seed deterioration, an inexorable and irreversible process, is mainly influenced by relative humidity and temperature, and is known to vary among species and seed populations.

The effects of relative humidity and temperature of the seed storage environment on seed life are highly interdependent (Copeland and McDonald 2001; Phaneendranath and Funk 1981). It is generally recognized that dry environment, moderate and uniform temperature, and partial or total exclusion of oxygen favor seed longevity (Shull 1914). Most crop seeds lose their viability near 80% relative humidity and 25 to 30 C, but can be stored successfully at relative humidity ≤ 50% and temperatures ≤ 5 C (Toole 1950). Harrington (1972) suggested that each 1% increase in seed moisture and each 5 C increase in seed temperature independently reduce the life of the seed by 50%. However, for seeds stored at > 14% seed moisture this assumption does not hold true and seeds begin to show increased respiration, and fungal invasion that rapidly reduce their viability. The control of relative humidity, and its effect on seed moisture, is more critical than storage temperature for optimum seed storage conditions (Copeland and McDonald 2001). Low-moisture seeds store well at temperatures up to 25 C, but high-moisture seeds will store well only if the

temperature is reduced to 10 C or less. High temperatures accelerate deterioration of high-moisture seeds by increasing metabolic activity (Copeland and McDonald 2001). In a study of the effect of storage conditions on germination of Kentucky bluegrass (*Poa pratensis* L.), seed stored at 21 C had greater loss in viability than that stored at 5.6 C for any given relative humidity (e.g. 96%) (Phaneendranath and Funk 1981).

Seeds of some species are capable of remaining viable for longer periods than others under similar conditions. Seeds of many species are not only unable to germinate in water, but gradually lose their viability and finally die (Hegarty 1978; Kidd and West 1919; Morinaga 1926b). However, Shull (1914) found that seeds of 24 of 58 species germinated after being kept in mud in a jar for 130 d in a cool room. In a longer experiment, 3 of 22 species germinated after 7 yr of continuous submergence in water in jars kept in a dark room with little temperature fluctuation. The grass that remained viable for the longest period was nimblewill (*Muhlenbergia diffusa* Schreb.), which germinated after 4.25 yr of continuous submergence (Shull 1914).

Several studies in the early 1900s analyzed the effect of water, desiccation, and temperature on seed germination in order to develop a method to increase germination at planting known as priming (Chippindale 1934; Kidd and West 1918, 1919; Levitt and Hamm 1943). Most often, germination is hastened after seed hydration, especially at low temperatures (Allen and Meyer 1998), but there are differences in the effects of seed soaking on germination amongst species (Chippindale 1934; Kidd and West 1918). Chilling imbibed seed at 4 C for

a minimum of 14 d is considered effective in breaking switchgrass (*Panicum virgatum* L.) dormancy (Beckman et al. 1993). Haferkamp et al. (1994) reported that even though no seeds of Japanese brome (*Bromus japonicus* Thunb.) germinated during a 10 d 0/10 C 12 h light chilling period, they did germinate rapidly when moved to a 8/23 C 12 h light regime. In general, soaking seeds may speed up germination, but deleterious effects are observed when the soaking period is too long (Kidd and West 1918; Tilford et al. 1925). Germination potential of kidney bean (*Phaseolus vulgaris* L.) decreased from 97 to 15% after 126 h of soaking in sterile water at 20 C (Tilford et al. 1925). Also, the beneficial effect of soaking may be counteracted by damage or induction of secondary dormancy upon dehydration (Hegarty 1978).

Dormancy is defined as those instances when, even though the environmental conditions are adequate for a seed to germinate, there is an impediment within the seed itself that must be overcome for germination to take place (Baskin and Baskin 2004; Eira and Caldas 2000; Simpsom 1990; Vleeshouwers et al. 1995). Secondary dormancy, which occurs after seed detachment and the loss of primary dormancy, can be induced in some species by certain environmental conditions and may be relieved when the environment is more favorable (Forcella 1998; Hilhorst 1998). Some fully hydrated seeds can remain viable and dormant in the soil for many years (Hegarty 1978). Temperature and, to a lesser extent, water potential play a predominant role in the regulation of secondary dormancy (Hilhorst 1998) and the control of cyclic seasonal changes in the dormancy status of seeds (Allen and Meyer 1998).

Chilling has been reported to induce secondary dormancy in winter annuals (Baskin and Baskin 1981) and in some populations of the perennial herb blue flax (*Linum perenne* L.) (Meyer and Kitchen 1994). Conn and Farris (1987) reported induction of secondary dormancy in wild oats (*Avena fatua* L.) after 9 mo burial at 2 and 15 cm under the cold conditions of Fairbanks, AK. The capacity of seeds to remain dormant during considerable periods of time prevents the extinction of an ecotype under temporally unfavorable conditions and enables the seed to take advantage of any event which restores conditions favorable for growth, allowing for potential establishment in new environments (Allen and Meyer 1998).

Creeping bentgrass is a perennial turfgrass that has extremely small seed (13,500 seeds g^{-1}) with a relative storability index of 3 (scale 1: low to 3: high), implying that 50% of the seeds are expected to germinate after five or more years of storage (Justice and Bass 1978). Harrington (1972) summarized reports that indicated that bentgrass seed had >50% germination after 11 years of dry laboratory storage. Hancock (2004) reported germination of transgenic glyphosate-resistant creeping bentgrass seed to be 31 to 78% and 2 to 8% after 6 and 24 mo burial at Corvallis, OR, respectively.

One of the main concerns about the deregulation of transgenic glyphosate-resistant creeping bentgrass is gene flow. Because of their reduced size, creeping bentgrass seeds can be easily moved by wind or water thus establishing plants outside of cultivation, especially when close to water sources such as creeks, rivers and irrigation canals, contributing to gene flow. There was a need to determine the impact of soaking time and water temperature on creeping

bentgrass seed germination potential and longevity in order to understand the potential of water as a means of seed movement and its impact on gene flow. As part of a study of gene flow from transgenic glyphosate-resistant creeping bentgrass (Zapiola et al. 2008), we assessed the reproductive capacity of seeds from creeping bentgrass panicles that were kept in water and the relevance of seed movement via water as a means of gene flow. Our hypothesis was that seed germination and viability would decrease as panicles were soaked in water for longer periods, and that the decrease would be greater at higher water temperature due to seed deterioration. The objectives of this study were to: 1) determine under laboratory conditions the effect of panicle soaking time, water temperature, and their potential interaction on seed germination and viability using three different creeping bentgrass cultivars; and 2) evaluate the rate at which panicles can be moved by water in irrigation canals.

MATERIALS AND METHODS

Plant Material and Treatments

Panicles were collected from three commercial fields of non-transgenic creeping bentgrass of the cultivars ‘Penncross’, ‘Penn A-4’, and ‘Penn G-2’, just before swathing on July 25, 2007, and were kept in paper bags at room temperature for 7 mo. The number of florets per panicle was quantified for 10 panicles of each cultivar. Each of two random subsamples of 50 panicles of each cultivar was placed in one of six sealed 11.4 L containers[1] with 5 L distilled water. One set of three containers, one of each cultivar, was kept at 20 C, and another set of three was kept at 4 C, both under 12 h light. Chosen temperatures reflect expected water temperature during late summer, early fall and the expected water temperature once the secondary canals start to freeze in late fall.

Six panicles were removed from the water after 1, 2, 4, 6, 8, 12, and 17 wk for each cultivar and temperature. Some seeds germinated in the water while still attached to the panicles and were counted and separated from each of the panicles removed from the water. Panicles were allowed to dry for 2 d on paper towels and then threshed by hand. Six non-soaked panicles were used as a control for each cultivar and temperature (0 wk).

Germination Test

Standard germination tests were performed with the threshed seed. Five replicates of fifty seeds each per treatment combination were placed on blotting

paper[2] in 9 cm Petri dishes with 10 ml distilled water in a growth chamber at 15/25 C and 12 h light. Petri dishes were placed in sealed polyethylene bags to prevent desiccation. Germinated seedlings were counted at 7 and 28 d. Seeds were considered germinated when both the radicle and the coleoptile were visible. Germination was expressed as a proportion of the 250 seeds. The experiment was repeated. Remaining threshed seeds were stored in paper envelopes at room temperature (ca. 20 C) under dry conditions. After 39 wk (9 mo) of dry storage, germination of seeds from panicles removed from the water at 17 wk and that of the 0 wk controls was determined using five replicates of 25 seeds each for each cultivar, temperature, and run. Simultaneously, viability of the seeds from the 17 wk at 4 C soaking treatment, and that of the 0 wk controls for each cultivar and run was determined for 4 replicates of 25 seeds each, using the tetrazolium (TZ) test at the Oregon State University Seed Laboratory. Germination also was determined for seeds of remnant panicles that were left in water for 11 mo using five replicates of 10 to 50 seeds, based on seed availability.

Panicle Float

Thirty panicles from each cultivar were weighed, measured from the insertion of the first panicle branch to the tip (panicle length) and from the end of the rachis to the tip (total length). Six panicles per cultivar were dropped at each of three locations in an irrigation canal in central Oregon, August 30, 2007. The distance each panicle traveled in 2 min was recorded using a measuring wheel[3] and a GPS unit[4] and is reported in m min^{-1}. Site 1 (44°41’23” N; 121°7’41’’ W) and

site 2 (44°41’51’’ N, 121°7’22’’ W) were on a main irrigation canal, and site 3 (44°44’29’’ N, 121°11’1’’ W) was on a secondary irrigation canal. The study was repeated September 12, 2007. Two panicles were dropped September 12, 2007, at a fourth location (44°40’45” N, 121°8’32” W) on a main canal and were followed for 1 hr 40 min. Panicles were recovered from the water. Air temperature, relative humidity, wind speed, water temperature at 5 cm, canal depth and width were recorded. An estimate of the water flow for the days of the study was obtained from the North Unit Irrigation District office in Madras, OR, (Bob Ringering, personal communication).

Statistical Analysis

Germination data were transformed using arcsin transformation to satisfy the homogeneity of variance. Transformed data were analyzed using a split/split randomized block design with water temperature as a main plot (2 levels), cultivar as a subplot (3 levels), time in water as a sub subplot (8 levels), and each run of the experiment was considered a block (2 levels) using PROC MIXED in SAS[5]. Means were compared using the Tukey-Kramer adjustment in SAS. Results presented are the non-transformed germination means (Ahrens et al. 1990). Viability (TZ) test results for the 17 wk at 4 C treatment and the 0 wk check were analyzed using PROC ANOVA in SAS. The effect of 9 mo of dry storage on germination of seeds from panicles soaked for 17 w at 4 and 20 C and the 0 wk check was analyzed using PROC ANOVA. Means were separated using confidence intervals. The panicle float data were analyzed as a completely

randomized factorial design with two factors (Date and Site) using PROC GLM in SAS.

RESULTS AND DISCUSSION

Seed Germination

There was a water temperature effect as well as a time in water effect on seed germination (Table 5-1). However, because the interaction between water temperature and time in water was significant, the results are reported based on the interaction (Fig. 5-1). There was no effect of experimental run on seed germination. There was no effect of cultivars or an interaction between cultivar and any other factor, indicating that the three cultivars used in this study responded in the same way to the soaking time and water temperature interaction.

When panicles were kept for 1 wk in the water, germination was not affected, regardless of the water temperature. At 2 wk and onwards, seeds from panicles soaked at 20 C had greater germination than those from panicles soaked at 4 C. Germination was 93% for the control (0 wk in water) and at 17 wk was 88% and 46% for the 20 C and 4 C treatments, respectively.

Germination decreased more in seeds from panicles kept in water at 4 C than at 20 C. There was no difference in germination between soaking times within the 20 C treatment, except at 4 wk (97%), when germination was greater than that at 1 wk (85%), but none of them were different from the 0 wk control. Within the 4 C treatment, germination was lower than the control for all soaking times, except for 1 wk (84%). While there was no difference between 2 wk (73%) and 4 (70%), 6 (59%), 8 (53%) and 12 wk (55%), there was a tendency of seed

Table 5-1. Analysis of variance for the effects of water temperature (Temp), cultivar (Cultivar) and time in water (Time) treatments on germination.

Source	DF	Sum of Squares†	Error DF	F value	Pr > F	Significance
Temp (A)	1	2.294	1	5240.37	0.0088	**
Cultivar (B)	2	0.017	4	1.4	0.3455	NS
A x B	2	0.018	4	1.47	0.3325	NS
Time (C)	7	0.909	42	11.59	<0.0001	***
A x C	7	0.802	42	10.23	<0.0001	***
B x C	14	0.093	42	0.59	0.8548	NS
A x B x C	14	0.103	42	0.65	0.8037	NS
Run	1	0.032	1	72.71	0.0743	NS
Run x A	1	$0.44\ 10^{-3}$	4	0.07	0.8041	NS
Run x A x B	4	0.025	42	0.56	0.6955	NS
Residual	42	0.471				

† Analysis corresponds to arsine transformed data.

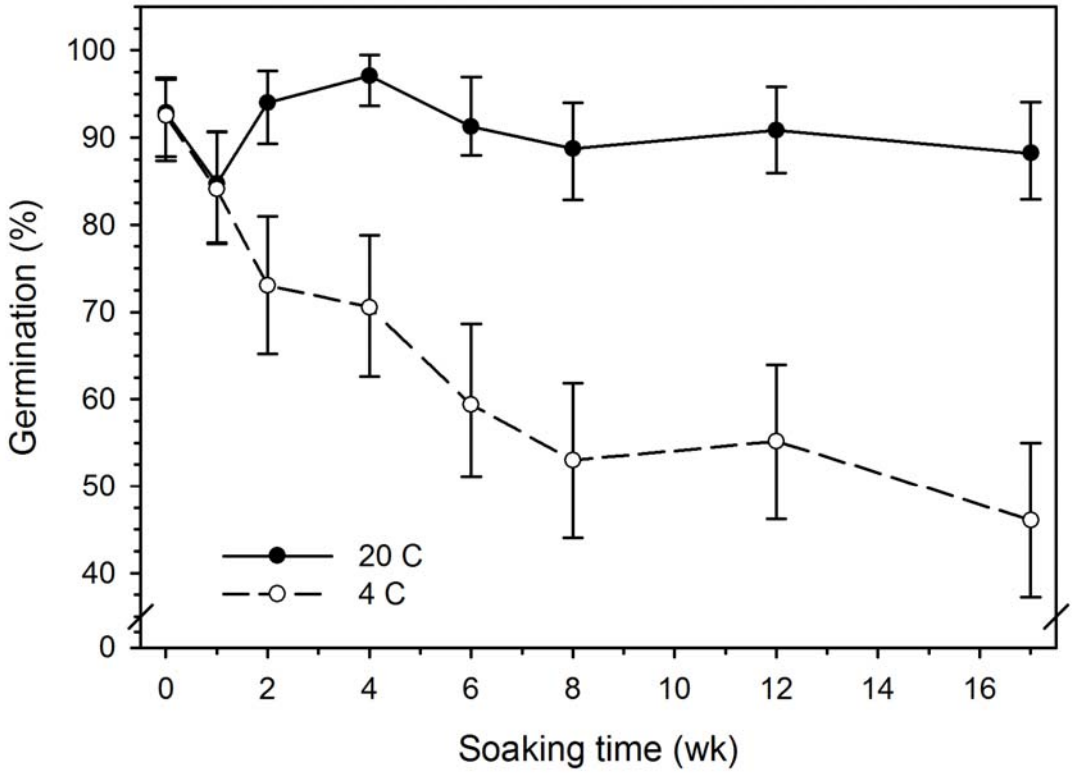

Figure 5-1. Interaction of soaking time (0, 1, 2, 4, 6, 8, 12, and 17 wk) and water temperature (20 C (●) and 4 C (○)) on creeping bentgrass seed germination, across cultivars. Data points are the original means for each treatment combination. Bars are the back-transformed limits of the confidence intervals for the arcsine means.

germination to decrease as the panicles were kept longer in the water. Although not different from that at 6, 8, and 12 wk, germination after 17 wk of soaking at 4 C was 46%.

Results of this study support the statement of Kidd and West (1919) that describes the effects of soaking seeds in water to be complex and highly dependent on the conditions under which the soaking of the seeds takes place. Although our hypothesis was that seed germination and viability would decrease as panicles were soaked for longer periods, and that the decrease would be greater at higher water temperature due to seed deterioration, we found the opposite. Germination of seeds from panicles kept for 17 wk in water at 20 C did not decrease. Even though germination of the seeds from the panicles soaked at 4 C was reduced, some seeds were still able to germinate after 17 wk of soaking. However, reduced germination does not imply that the seeds were not viable. We hypothesized that soaking seeds at 4 C, a cold temperature, induced secondary dormancy which was responsible for the decreased germination after being in water.

Dormancy is gradual and reduces the window of temperatures that allow seed germination (Vleeshouwers et al. 1995). Therefore, if seeds have limited germination under optimal conditions, they can be considered to be dormant (Baskin and Baskin 2004). Dry storage at room temperature has been found to release dormancy in wild oat (Chen and Varner 1973). To further explore the hypothesis of secondary dormancy induction, viability and germination tests were

conducted on seed remaining from the 0 and 17 wk soaking treatments that were then kept dry at room temperature for 9 mo.

After 9 mo of dry storage, viability of the seed from panicles previously soaked at 4 C for 17 wk (92.5%) was comparable to that of the non-soaked (90%) based on the TZ test results (p=0.18), confirming that soaking at 4 C for 17 wk did not kill the seed. Germination results after 9 mo of dry storage of the previously soaked and the non-soaked seed were compared with the results obtained at the time seeds were removed from the water (17 wk) and those of the check (0 wk), to determine how germination of both soaked and non-soaked seed was affected by dry storage. There was an interaction between dry storage (before and after) and soaking treatment (0 wk, 17 wk at 20 C, and 17 wk at 4 C) on germination (Table 5-2). There also was an interaction between cultivar and soaking treatment on seed germination, implying that different cultivars responded differently to 0 wk and 17 wk soaking treatments, regardless of dry storage.

Nine months of dry storage at room temperature in the laboratory (ca. 20 C) increased germination of seed from panicles soaked for 17 wk at 4 C from 46 to 86% (Fig. 5-2). However, while germination after dry storage of seeds soaked for 17 wk at 20 C (98%) was no different from that of the non-soaked seeds (98%), germination of seeds soaked for 17 wk at 4 C was still lower than that of non-soaked ones. Although there was no difference between germination at 0 wk (93%) and 17 wk at 20 C (88%) before dry storage, 9 mo of dry storage increased germination of the 17 wk at 20 C treatment and had no effect on that of the 0 wk.

Table 5-2. Analysis of variance for the effects of 9 mo of dry storage (Dry storage), soaking treatment (Soaking) and cultivar (Cultivar) treatments on creeping bentgrass germination.

Source	DF	Sum of Squares	F value	Pr > F	Significance
Run	1	181	4.06	0.0456	*
Dry storage (A)	1	15,080	338.09	< 0.0001	***
Soaking (B)	2	31,842	356.93	< 0.0001	***
A x B	2	10,672	119.63	< 0.0001	***
Cultivar (C)	2	344	3.86	0.0231	*
A x C	2	20	0.23	0.7950	NS
B x C	4	1056	5.92	0.0002	**
A x B x C	4	133	0.75	0.5620	NS
Residual	161	7181			

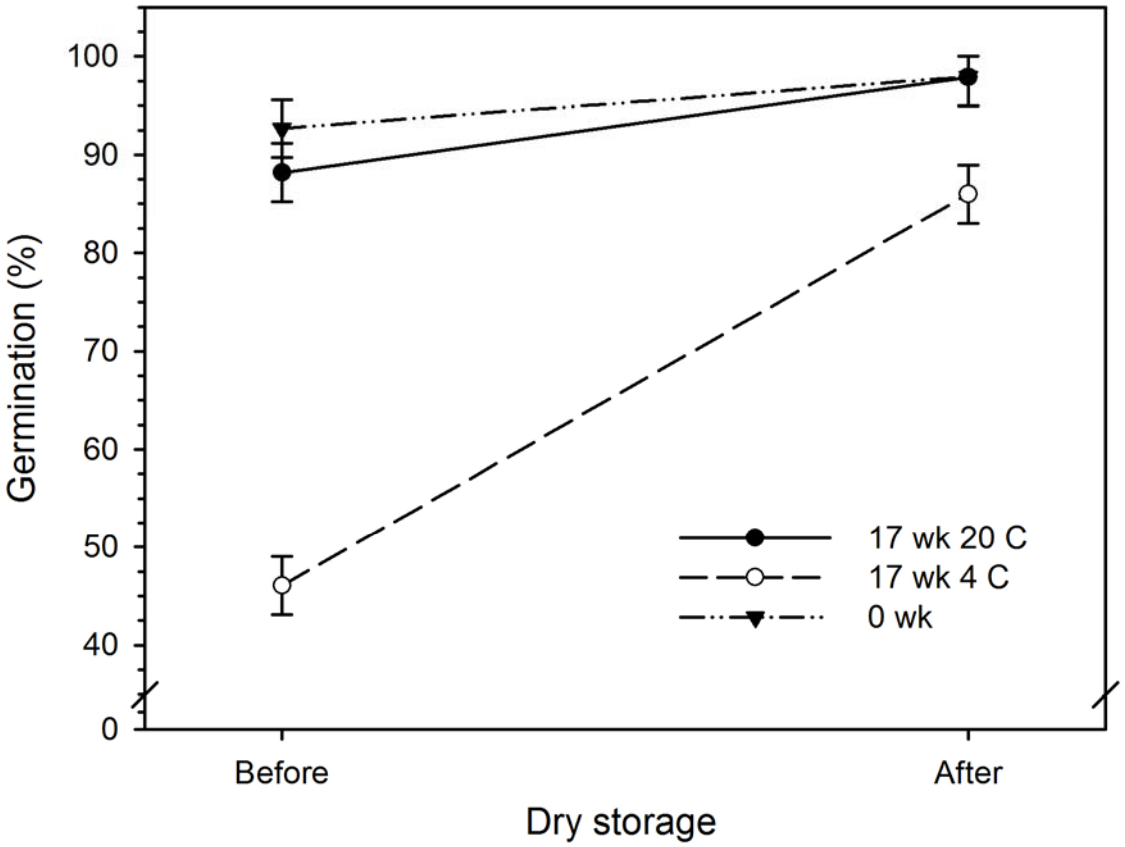

Figure 5-2. Interaction of dry storage (before and after) and soaking treatment (17 wk at 20 C (●), 17 wk at 4 C (○), and 0 wk (▼)) on creeping bentgrass seed germination, across cultivars. Data points are the original means for each treatment combination. Bars are the limits of the confidence intervals.

While there was no difference in germination between cultivars for the 0 wk and 17 wk at 20 C soaking treatments, Penn A-4 was affected more by 17 wk of soaking at 4 C than were Penn G-2 and Penncross (Fig. 5-3). This result suggests that subtle differences between cultivars not distinguished when considering all soaking times and temperature combinations may be magnified beyond 17 wk of soaking. However, this difference between Penn A-4 and the other cultivars was not seen in the response to dry storage, implying that 9 mo of dry storage had the same effect in overcoming secondary dormancy for all cultivars, regardless of their germination baseline.

The fact that, when tested at the optimal germination temperature for creeping bentgrass of 15-25 C (AOSA 2002; Toole and Koch 1977), seed soaked for 17 wk at 4 C had lower germination at the time of removal from the water than when tested again after being in dry storage at room temperature for 9 mo supports our hypothesis of secondary dormancy induction. If the seeds were not at optimal germination conditions, it could be argued that the environment inhibited germination. However, after 1 wk of being soaked in water at 4 C, some seeds entered a state where they no longer germinated, even under the optimum environment. This superimposed condition preventing seeds from germinating when placed in an optimal environment is known as secondary dormancy. Simpson (1990) reported that excess of water can induce secondary dormancy in grasses. However, we did not see reduced germination in the seeds from panicles soaked at 20 C when compared with the 0 wk treatment. Maguire (1969) indicates that bentgrass is a species with distinct endogenous rhythms that control

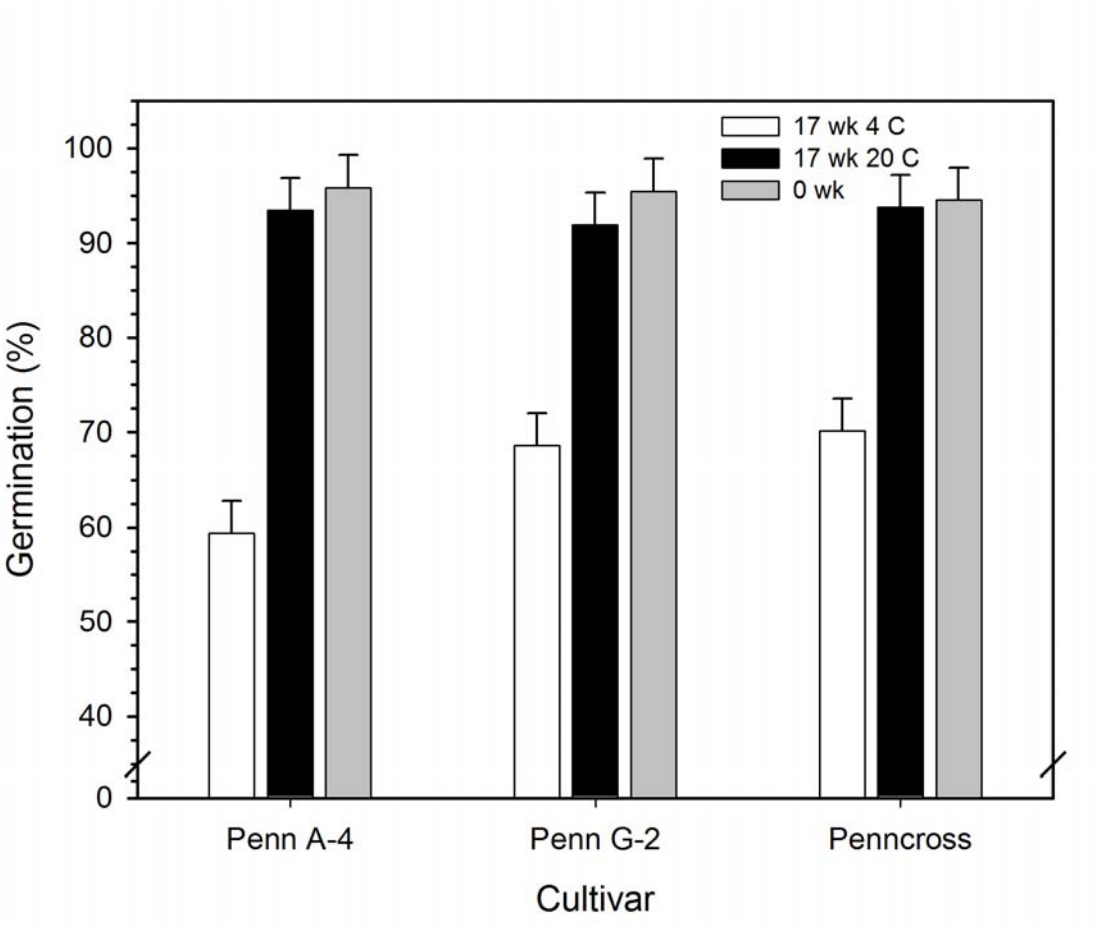

Figure 5-3. Interaction of Cultivar (Penn A-4, Penn G-2, and Penncross) and soaking treatment (17 wk at 4 C (white), 17 wk at 20 C (black), and 0 wk (gray)) on creeping bentgrass seed germination, across dry storage. Data points are the original means for each treatment combination. Bars are the upper limits of the confidence intervals.

germination independently of storage conditions. Because seed germination was affected by the storage conditions, this rhythmic phenomenon was not the cause of the reduced germination for the seeds soaked at 4 C.

These results imply that low temperature played a decisive role in the induction of secondary dormancy in soaked creeping bentgrass seed and that 9 mo dry storage at room temperature overcame most of the secondary dormancy that was imposed by the moist-chill treatment. Once the secondary dormancy was at least partially overcome after 9 mo of dry storage, seeds had greater germination when placed under the same conditions where they failed to germinate when removed at 17 wk from the water at 4 C. Our findings raise the interesting questions of whether the results would have been the same if the panicles were kept dry at 4 C instead of soaked, kept in the dark, or if instead of constant temperature, alternating 4 and 20 C were used during soaking.

Secondary dormancy can be induced at different points in the germination process in both dry and imbibed seed (Eira and Caldas 2000). In this study, dormancy was clearly induced after 1 wk soaking in cold water because there was no difference in germination from the control at that point, regardless of water temperature. Because seeds germinated less while in the water at 4 C than they did when soaked at 20 C (data not shown), we speculate that secondary dormancy was induced while the seeds were in the water at 4 C and not upon dehydration.

Even though we did not use transgenic material for these experiments, Elias et al. (2009) showed that viability, vigor and potential longevity of transgenic accessions were similar or lower than those of non-transgenic cultivars.

Because it was not part of the planned experiment and was, therefore, just based on germination of seeds from remnant panicles that were left in the water for 11 mo, we did not include the results of the germination after 11 mo of soaking in our analysis. Nevertheless, it is worth noting that germination of seeds from panicles kept in water for 11 mo ranged from 47 to 100%, with an average on 78%, across water temperatures.

Panicle Float

There was an interaction ($p< 0.0001$) between date and site for distance travelled per unit of time in the panicle float study. This interaction reflects the complexity of the factors involved, such as wind direction, wind speed and water flow (Table 5-3). The sites were chosen to represent different types of waterways in the North Unit Irrigation District in central Oregon. We concluded that a panicle which falls in an irrigation canal can travel between 7.5 and 29 m min^{-1} with an average of 19 ± 1 m min^{-1} (Table 5-4). This result is in agreement with the 2 km that the two panicles travelled in average in 1 h 40 min (20 m min^{-1}) at the fourth site on September 12, 2007, in a canal with a water flow of 169.8 m^3 min^{-1}. These findings can be used as a starting point for further modeling studies. Considering that the panicles had an average of 345 ± 69 (range 87 to 782) florets, and that

Table 5-3. Description of the panicle float sites.

Date	Conditions	Site 1	Site 2	Site 3
8/30/2007	Air temperature (C)	30	32	27
	Relative humidity (%)	21	21	39
	Wind speed(km h^{-1})	10	16	15
	Wind direction	NW	NW	NW
	Canal width (m)	8.6	8.6	1.8
	Canal depth (m)	1.4	1.6	0.3
	Water flow (m^3 min^{-1})	132.5	125.7	0.8-1.7
	Water temperature (C)	23	23	25
9/12/2007	Air temperature (C)	31	30	25
	Relative humidity (%)	28	28	42
	Wind speed (km h^{-1})	5	7	7
	Wind direction	N	N	N
	Canal width (m)	8.6	8.6	1.7
	Canal depth (m)	1.4	1.6	0.2
	Water flow (m^3 min^{-1})	144.4	135.9	0.8-1.7
	Water temperature (C)	19	19	21

Table 5-4. Average, standard deviation, and range of distance traveled (m min^{-1}) by the panicles in the water for the two dates at each of the three sites.

	8/30/2007			9/12/2007		
	Average	SD	Range	Average	SD	Range
Site 1	23.1	3.5	17.2 – 28.3	22.1	3.0	14.8 – 26.0
Site 2	11.1	1.6	7.4 – 13.7	16.4	1.5	13.3 – 18.4
Site 3	16.1	3.3	8.1 – 19.7	24.2	4.7	14.4 – 28.9
Average	16.8	5.7	7.4 – 28.3	20.8	14.6	13.3 – 28.9

65% of those florets produced viable seeds, an average of 224 viable seeds per panicle could be carried in an irrigation canal 19 m min^{-1}.

Summary

In order to estimate the potential contribution of seeds to transgene movement and introgression, it is important to understand how soaking in water affects the germination potential of creeping bentgrass seeds. Creeping bentgrass seeds did not lose the potential to germinate after 17 wk in water at 20 C and, although reduced, germination was still 46% after 17 wk in water at 4 C. The reduction in germination for the seeds soaked at 4 C for 17 wk was caused by secondary dormancy, which was partially overcome by dry storage for 9 mo. Therefore, a seed that falls in an irrigation ditch/canal at 20 C has the potential to germinate and establish downstream for a considerable window of time. On the other hand, a seed that falls in cold water can be induced into secondary dormancy and be transported and deposited where it will remain dormant until the secondary dormancy is overcome. Based on our results, longer time experiments (longer than 11 mo) need to be conducted to determine if there is a significant decrease in germination when soaked at 20 C. These findings add an extra level of complexity to the study of transgene flow in creeping bentgrass and suggest that seed movement via water has to be regarded as an important means for gene flow in time and space.

SOURCES OF MATERIAL

[1] Sterilite® 11.4L, Sterilite Corporation, Townsend, MA.

[2] Hoffman Manufacturing Inc. Albany, OR.

[3] Road Runner RR318N outdoor long-run wheel, Keson Industries, Aurora, IL.

[4] GARMIN eTrexLegend, Garmin International Inc. Olathe, KS.

[5] SAS, Version 9.2, SAS Institute, Cary, NC.

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CAPTER 6: GENERAL CONCLUSIONS

This multiyear study of gene flow of a transgene at the landscape level has limitations on interpretation of the results and the conclusions that can be made, but it provides empirical evidence as to what happened and how the fate of the transgene in the environment evolved over the years. A single introduction of a transgenic event has long term impacts that can not be predicted by looking at only one year. The system is dynamic and influenced by many different factors.

The CP4EPSPS transgene escaped the GRCB production fields and established outside of cultivation, along irrigation canals, ditches and ponds, despite the strictly regulated production practices and the mitigation program put in place. Pollen load in the environment after the removal of the GRCB seed production fields was enough to contribute to gene flow the following years. Gene flow via pollen allowed the formation of interspecific and intergeneric hybrids, and gene flow through seeds contributed to spreading the new biotypes to locations where there were no creeping bentgrass the previous year.

The large difference between the frequency of pollen-mediated gene flow and the proportion of GR plants found *in situ* could be attributable to the fact that creeping bentgrass, redtop, and the interspecific hybrid are perennials. Therefore, each GR plant can contribute the transgene to the environment for more than one year. Also, the number of established plants can build up over time. A limitation we had during the survey was the impossibility to determine when a certain plant established in a particular location if that location was not surveyed the previous

year, unless the plant found was a seedling. Another factor that plays a role in affecting the proportion of GR plants is the glyphosate selection pressure. Transgenic GRCB has been found to only have a competitive advantage over conventional creeping bentgrass when glyphosate is sprayed. Growers and the irrigation district staff make frequent glyphosate applications on ditch and canals banks to control weeds, and that positive selection pressure can be a critical factor for increasing the proportion of GR plants.

Because reference material from the originally planted seed could not be obtained, we were not able to determine what percentage of the gene flow should be attributable to pollen and which one to seed, but we can conclude that both played a role in moving the transgene. In addition, despite the fact that it was not the objective of our study, we should not disregard the potential of gene flow through vegetative propagules (Dysart & Mallory-Smith 2006), which in the case of creeping bentgrass are stolons, but in the case of interspecific hybrids can be both stolons and rhizomes. Gene flow through vegetative propagules does not require pollen production, flowering synchronization or successful fertilization because there is no need to produce a viable seed in order to establish a transgenic plant. Further, the plant established does not need to be fertile to reproduce vegetatively. Vegetative propagation could allow interspecific hybrids with lower fertility levels to perpetuate in the environment.

Although the combination of *matK* indel and cpSSR chloroplast markers with nuclear ITS sequence was effective in confirming some intergeneric and interspecific hybrids, the polyploid nature of creeping bentgrass (2n=4x) and

redtop (2n=6x) requires the sequencing of many clones which is not practical or economically appealing when studying several plants.

We confirmed the production of interspecific (GR redtop x creeping bentgrass) and intergeneric (GR creeping bentgrass x rabbitfoot grass) hybrids *in situ*. But, the fact that creeping bentgrass, redtop and rabbitfoot grass are sympatric in central Oregon and have been there for some time, makes us suspect of the fact that, although we confirmed a susceptible creeping bentgrass x redtop hybrid *in situ*, we were not able to confirm a GR interspecific or intergeneric hybrid *in situ*. We wonder if some of the plants that have creeping bentgrass chloroplast and were grouped with the creeping bentgrass based on the ITS sequence but have rhizomes, which is considered a redtop morphological characteristic, are backcrosses to creeping bentgrass. More clones would have to be sequenced to reveal redtop traces.

The difficulty to distinguish species *in situ*, and the fact that we detected the generation of both interspecific and intergeneric hybrids in our screening, highlights the fact that *Agrostis* is a very complex genus, and that the definition of species as being “a group of actually or potentially interbreeding populations that are reproductively isolated from such other groups” is challenged. Future work will have to focus on developing a sound set of nuclear molecular markers to be combined with the *matK* and cpSSRs to aid in species and hybrid identification and facilitate the ongoing monitoring and mitigation processes, especially if GRCB never is deregulated.

The fact that creeping bentgrass seeds were able to germinate after such long period of being soaked in water was surprising. The proximity of creeping bentgrass plants to the waterways increases the likelihood of water-mediated GR seed dispersal. These results, combined with those of Hancock (2004), highlight the importance of considering seeds as a means of gene flow, in both space and time.

Ecologically, gene flow from transgenics could have an impact on the environment if the trait introduced, such as increased water or nutrient use efficiency, results in a competitive advantage versus the non-transgenic biotype or if the trait allows the transgenic plant to expand its ecological niche like in the case of drought or salt tolerance. However, if the same trait is achieved via conventional breeding, it could have the same environmental impact. From the weed management point of view, and specifically for herbicide resistant crops, the fact that the trait was incorporated via genetic engineering or conventional breeding will not change the potential for gene flow to relative feral or wild species and the economic impact it could have as a volunteer in another crop.

However, gene flow from transgenic plants may have some social, religious, cultural and public perception implications. Transgene flow definitely has marketing and economic implications, because it can compromise the sale of products if the transgene is detected in shipments to markets with zero transgene tolerance. Gene flow from transgenic crops has potential to impact the economy of a specialized production region and ruin its reputation worldwide. Globally accepted tolerance levels of GE material are needed for coexistence of GE and

non-GE crops, especially if outcrossing, wind-pollinated, small-seeded perennial grasses are to be deregulated.

This study shows that the increased isolation distances were not enough, and that they need to be revisited if GRCB is deregulated and is expected to coexist with non-transgenic cultivars. Furthermore, pollen and seed, especially in small-seeded crops, disperse too easily and too far, to make containment practical.

Even though some molecular strategies, such as pollen sterility (Luo et al. 2005), maternal inheritance, seed sterility among others (Daniell 2002) have been considered as an option to prevent gene flow, the fact that interspecific hybridization can occur with the transgenic plant as the pollen receptor, and produce seed, or in species such as creeping bentgrass, the transgene can be propagated vegetatively, does not seem to solve the problem of gene flow in outcrossing, perennial, vegetatively propagated species.

A limitation that is common to most gene flow studies is the difficulty to determine how far the last pollen grain/propagules traveled. And this case is not different. We will never know how far the last pollen grain/propagule went; therefore, the transgenic event GRCB will not be able to be removed from the environment. However, our findings should be considered when evaluating the use of genetic engineering technology in outcrossing, wind-pollinated, small-seeded, perennial crops, and when designing risk assessments for the release and deregulation of such GE crops.

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