Chapter 10
Gene Flow in Genetically Engineered Perennial Grasses: Lessons for Modification of Dedicated Bioenergy Crops

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10.1 Introduction

The potential ecological consequences of the commercialization of genetically engineered (GE) crops have been the subject of intense debate, particularly when the GE crops are perennial and capable of outcrossing to compatible relatives (Aldhous 2003; Colwell et al. 1985; Eastham and Sweet 2002; Giles 2003; Marvier

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and Acker 2005; Rogers and Parkes 1995; Tsuchiya et al. 1995). The ecological impact issues for engineered perennial crops are the following: whether (1) the techniques themselves or resulting phenotypic traits could lead to adverse ecological impacts; (2) escaped GE crop plants can persist in the environment via feral populations or hybridization with non-transgenic populations of the same or related species, depending on the source and nature of the GE trait(s) in the crop; (3) long-term environmental effects will result from commercialization of the GE crop (Eastham and Sweet 2002; Tiedje et al. 1989; Wrubel et al. 1992; Ellstrand and Hoffman 1990); (4) GE crops are grown sympatrically with wild relatives (e.g., centers of origin) or cross-compatible species (or genera); (5) GE crops have biotypes or related taxa that are already aggressive weeds; (6) GE crops can also be weeds themselves; and (7) GE crops can outcross with some degree of self-incompatibility. Most of the thousands of small-scale field tests of transgenic plants have not been designed to investigate the environmental consequences of gene flow associated with widespread commercialization (Dale et al. 2002; Eastham and Sweet 2002; Wrubel et al. 1992). However, more recent studies (Belanger et al. 2003; Christoffer 2003; Mallory-Smith and Zapiola 2008; Reichman et al. 2006; Watrud et al. 2004; Zapiola et al. 2008) demonstrate that commercialization of GE perennial grasses could lead to transgene flow via outcrossing with wild relatives and establishment of feral populations via seed escape, and may therefore present significant ecological and economic risks.

Numerous risk assessments have been conducted on transgenic plants of annual and/or self-pollinating crops (Belanger et al. 2003; Dale et al. 2002; Eastham and Sweet 2002; Ellstrand and Hoffman 1990; Ellstrand et al. 1999; Rogers and Parkes 1995). For instance, Eastham and Sweet (2002) reviewed the significance of, and the parameters affecting, gene flow in six major crop species including oilseed rape (*Brassica napus*), sugar beet (*Beta vulgaris*), potato (*Solanum tuberosum*), maize (*Zea mays*), wheat (*Triticum aestivum*), and barley (*Hordeum vulgare*). Each crop was reviewed with attention to the following points: (1) reproductive biology and crop use; (2) type of genetic modification; (3) pollen dispersal potential; (4) gene flow: crop-to-crop, including hybridization capacity and possible consequences of gene flow; (5) definition and status as a weedy species; and (6) gene flow: crop-to-wild-relative, including compatibility and distribution, hybridization, and gene flow. Using these parameters, their report focused on the significance of pollen-mediated gene flow in annual crops and provides relative risk assessments of gene flow from crop to crop and from crop to wild relatives.

However, it is also now recognized that seed-mediated gene flow is a major concern (Mallory-Smith and Zapiola 2008). Contamination of seed with non-deregulated crops, such as the StarLink and *Bt10* incidents in US maize seed, have caused serious commercial and economic impacts and negatively affected public perception and trust (Bucchini and Goldman 2002; Macilwain 2005). Grain from transgenic US corn is exported to many countries as living seeds, and this can create legal and social problems if transgenic seeds are planted in nations where they are not approved for environmental release, as occurred in Mexico. Transgenes were detected in open-pollinated landraces of corn in Oaxaca, Mexico, in 2000

(Quist and Chapela 2001) and again in 2001 and 2004 (Piñeyro-Nelson et al. 2009), although the number of locations with confirmed reports is quite low (Ortiz-García et al. 2005; Piñeyro-Nelson et al. 2009; Snow 2009). Thus, as Marvier and Van Acker (2005) note, the escape of transgenic seed via human error is quite likely, even in crops with large seeds such as maize.

It is clear that transgene flow depends on several variables: the specific crop, its location, the presence of outcrossing wild relatives/sexually compatible crops, and the fitness effect(s) of the GE trait (Daniell 2002). It is also clear that the mechanisms responsible for gene flow among crops and their related and wild relatives are: (1) dispersal of viable pollen; (2) dissemination in seed; or, in some cases (3) vegetative dispersal, e.g., by means of stolons in some perennial grasses. The major vectors for dispersal are considered to be largely wind, water, animals, and human activities.

Gene flow research is especially important in species with a high propensity for outcrossing or gene introgression. Recent studies highlight the potential for gene flow from the commercialization and large-scale seed production of perennial transgenic grasses (Mallory-Smith and Zapiola 2008; Reichman et al. 2006; Watrud et al. 2004; Wipff and Fricker 2001; Zapiola et al. 2008). Perennial grasses are grown throughout the world; furthermore, the vegetative and reproductive biology of many plants targeted for bioenergy production such as the perennial grasses switchgrass (*Panicum virgatum*) and big bluestem (*Andropogon gerardii* Vitman), and trees such as poplar (*Poplus spp*.), willow (Salix spp.), and *Paulownia* makes some gene flow to wild species or the environment inevitable. We need to come to an understanding regarding the limitations of the technologies used to mitigate gene flow and what constitutes an acceptable level of escape. Towards these ends, a review of the science of gene flow in GE perennial grasses is presented here.

10.2 Gene Flow in Glufosinate-Resistant Grasses

Field studies have been conducted to assess pollen-mediated gene-flow using open-pollinated transgenic glufosinate-resistant grasses (Bae et al. 2008; Belanger et al. 2003; Wipff and Fricker 2001) and have clearly demonstrated gene flow to non-transgenic grasses. The first of these studies (Wipff and Fricker 2001) was conducted in the Willamette Valley in Oregon, using 286 creeping bentgrass plants that were transformed with the *bar* gene, which confers resistance to glufosinate ammonium herbicides (i.e., Basta^{Tm}, Finale^{Tm}, Liberty^{Tm}). This field study was conducted under the authority, guidelines, and provisions provided by United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) Biotechnology Regulatory Services. The objectives of the study were to (1) gather initial data on pollen movement; (2) test the effectiveness of cereal rye (*Secale cereal* L.) borders as a pollen barrier, which were used successfully in the isolation of tall fescue and perennial ryegrass nurseries; (3) study interspecific gene flow into four introduced species of bentgrass; and (4) verify the fertility of the

transgenic bentgrass plants. The results of that study demonstrated that pollen from the transgenic nursery traveled at least 300 m. The most distant recipient plan, on the SW transect, had 15 seedlings survive glufosinate applications. Polymerase chain reaction (PCR) and Southern blot analyses have confirmed the presence of the *bar* gene in these individuals.

Belanger et al. (2003) measured the frequency of interspecific hybridization by pollen-mediated gene flow between transgenic creeping bentgrass (*Agrostis stolonifera* L.) and four related species: velvet bentgrass (*A. canina* L.), dryland bentgrass (*A. castellana* Boiss & Reuter), redtop (*A. gigantea* Roth), and, colonial bentgrass (*A. capillaris* L.). The transgenic creeping bentgrass plants used in this study expressed the *bar* gene. They examined two identical transgenic plots, spatially separated by 140 m, each consisting of a hexagonal array including 90 sample points for pollen reception and a central point for pollen dispersal. The center pollen dispersal array consisted of five transgenic plants and the distance between each sample point was 3 m. At each sample point, five pollen recipients were placed using one plant each of the four related *Agrostis* species and one non-GE *A. stolonifera* plant to provide an indication of where in the plot the transgenic pollen was available. Interspecific hybridization occured between transgenic creeping bentgrass and both dryland bentgrass and colonial bentgrass (at frequencies of 0.04 and 0.002%, respectively), but no hybrids were recovered between GE creeping bentgrass and velvet bentgrass or redtop. The intraspecific hybridization resulting from pollination with nontransgenic creeping bentgrass was significantly higher (0.63%) The size of these plots and the number of transgenic plants involved did not approach real world commercial plots, which disperse far greater loads of transgenic pollen that would have the capability of traveling much greater distances. However, this design presents an excellent model to examine pollen-mediated gene flow, hybridization frequencies and seed scatter using male-sterile plants as recipients at the center.

The results from the studies above established that: (1) intraspecific gene flow in creeping bentgrass is possible for much longer distances than traditionally calculated; (2) transgenes can flow considerable distances to other species of *Agrostis* (i.e., interspecific gene flow) probably via pollen; (3) the transgenic hybrid bentgrass plants are fertile and stable; and (4) neither cereal rye or spatial separation provide an effective pollen barrier for confinement. These studies also strongly implicate pollen-mediated gene flow from male-fertile open-pollinated plants as a major obstacle to transgene confinement. However, neither the *Agrostis* or *Zoysia* studies that utilized the glufosinate resistance marker (the bar gene), addressed the possibility of transgene escape via seed shatter and dispersal by wind or other abiotic or biotic means.

The potential for intra- and inter-specific hybridization via pollen-mediated gene flow from transgenic *Zoysia* grass (*Zoysia japonica* Steud.) carrying the *bar* gene to wild type (WT) *Zoysia* grass and 14 weed species was investigated from 2003 to 2005 in Nam Jeju County, Korea (Bae et al. 2008). A number of experimental plot designs were deployed to detect gene flow; in addition, 121 sites up to 3 km outside the perimeter of the 936 m^2 GE test field were screened for potential hybrids and

seed escape. The authors reported significant intraspecific hybridization within distances of <3 m (6% hybrid seeds, SE = ±4%), but found that hybridization frequency effectively dropped to zero at distances greater than 3 m. There were no reported cases of interspecific hybridization with co-habitant weed species and no evidence was found for gene flow via pollen or seed from the experimental field, at least at the 121 external sites tested. The authors noted that a number of factors played a role in the above results, including: (1) *Z. japonica* is an inherently recalcitrant cross pollinator; (2) *Zoysia* seeds exhibit a very low germination rate (4%) after winter dormancy under natural conditions; and (3), the GE pollen source was relatively small. Thus, they conclude that while long distance gene flow is of lesser concern in GE *Zoysia* than in a highly outcrossing species such as creeping bentgrass further gene flow studies using larger plots of GE *Zoysia* grass are justified.

10.3 Gene Flow in Glyphosate-Resistant Creeping Bentgrass

In late 2002, under USDA-APHIS regulated status, 162 ha of a Round Up® Ready bentgrass variety (*Agrostis stolonifera L.*) were planted by The Scotts Company (http://www.scotts.com/) under permit within a 4,553 ha control area in central Oregon. An additional 2.4 ha field planted in 2003 flowered and produced seed in 2004 (Zapiola et al. 2008). This turfgrass variety contained the EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene from *Agrobacterium tumefaciens* strain CP4 and is the first example of a transgenic perennial grass crop to attempt passage through the regulatory process. An APHIS preliminary risk assessment concluded that the genetically engineered line (ASR368) used in the study was not significantly different from its parental line or null comparators except for its tolerance to glyphosate and is not sexually compatible with any threatened or endangered species or any species on the Federal Noxious Weed List (http://plants.usda.gov/java/noxiousDriver#federal).

Glyphosate-resistant creeping bentgrass (GlyRCB) was chosen as a commercial target for use on golf courses because of its good stand persistence even with repeated close mowing, and the herbicide-tolerance trait was expected to enable better weed control. The type of end use management for GlyRCB should ideally minimize gene flow via pollen and make seed development unlikely. The 162 ha planting was comprised of eight spatially isolated fields of varying sizes, presenting a unique large-scale testing opportunity to monitor gene flow in a genetically engineered perennial grass prior to its release as a commercial product.

This experimental cultivation raised concerns among many grass seed producers and breeders in the Willamette Valley of western Oregon, which is the site of 70% of US grass seed production. Creeping bentgrass is largely self-incompatible, highly outcrossing, and wind pollinated. It can hybridize with compatible species and reproduce by vegetative stolons that can persist and propagate outside of cultivation. The issue of creeping bentgrass seed size comes up at least three times, each with a slightly different presentation of the numbers. Seems redundant

(AOSA 2002), and the mean creeping bentgrass seed yield in Oregon is 600 kg ha^{-1}, roughly 8.1×10^9 seeds (USDA-NASS 2006). Another concern was the potential production of RoundUp® resistant weeds because control of escaped plants is difficult as alternatives to RoundUp® may be less effective, more expensive, or not for that use. It was also unclear who would finance the registration of alternative herbicides if contamination occurred. Furthermore, contamination of other harvested crops with transgenic seed is a serious marketing issue, especially in domestic and international markets that are not open to GE crops (Zapiola et al. 2008).

In response to these concerns, a 4,553-ha control district was established by the Oregon Department of Agriculture in Jefferson County, OR. This control district was intentionally located >150 km east of Oregon's Willamette Valley grass seed production area with some of the following requirements: (1) non-GE *Agrostis* ssp. could not be grown, planted, or handled within the control district; (2) field borders, ditch banks, and roadsides within 50 m of the GlyRCB production fields were to be kept free of *Agrostis* ssp.; (3) GlyRCB fields were located more than 400 m away from any creeping bentgrass field outside the control district (Zapiola et al. 2008). Additional safeguards implemented to prevent seed movement included transport of seed in sealed containers to and from fields, cleaning of equipment prior to leaving the field, use of dedicated combines for the GE crop, burning of straw remaining in the field to destroy any seed left behind, and cleaning and packaging of seed produced in the control district within the same area. Thus, several precautions were to be taken to help prevent seed scatter from the Round Up® Ready production fields.

10.3.1 Gene Flow via Pollen in Glyphosate-Resistant Bentgrass

More than 30 species of *Agrostis* occur in North America, and approximately one dozen species are found in Oregon (http://plants.usda.gov). Creeping bentgrass, redtop, colonial bentgrass, dryland bentgrass, velvet bentgrass, and brown bentgrass (*A. vinealis* Schreber) form a hybridizing complex of inter-pollinating, cross-compatible species. Although naturally occurring F_1 hybrids of *Agrostis* may exhibit reduced fertility or even sterility, pollen can remain viable for 2 h, and under optimal conditions, fertile hybrids can be formed; backcrossing can restore fertility in full (Belanger et al. 2003; Fei and Nelson 2003; Pfender et al. 2007). Thus, gene flow from GlyRCB production fields to populations of *Agrostis* spp., and the establishment of feral glyphosate-resistant populations has long been a distinct possibility.

Two groups, one led by Carol Mallory-Smith at Oregon State University, and the other by Lidia Watrud at the US Environmental Protection Agency (EPA) monitored gene flow from the production fields. Outside the control district, 69 resident *Agrostis* as well as 178 "sentinel" creeping bentgrass plants were used in 2003 by the US EPA to monitor gene flow via pollen from the eight GE test fields

(Reichman et al. 2006; Watrud et al. 2004). Based on testing of seedlings in the greenhouse, these studies detected pollen-mediated gene flow at much longer, i.e., landscape level distances, measured in kilometers, rather than much shorter distances (typically measured in meters), as reported earlier. While the highest relative frequencies of gene flow were observed within 2 km of the control area perimeter, *CP4 EPSPS*-positive seedlings were recovered from resident creeping bentgrass and redtop, and sentinel creeping bentgrass at maximal distances of 8, 14 and 21 km, respectively. In 138 sentinel creeping bentgrass plants tested, 75 plants yielded positive seedling progeny (54%) and the overall incidence of *CP4 EPSPS* positive seedlings was 2.0% (625 positive / 32,000 total seedlings tested). Of the 30 resident (i.e., wild) creeping bentgrass plants, 16 also yielded positive seedling progeny (53%), and 157 positive seedling progeny of 565,000 tested (0.03%) were obtained. Resident redtop also produced glyphosate-resistant progeny, with 13 positive of 39 tested (33%); the overall incidence of positive seedlings (159 positive/397,000 seedlings tested) was 0.04%. Molecular confirmation of the presence of the *CP4 EPSPS* gene in all positive seedling progeny was obtained via PCR amplification; the 1,050 bp PCR product sequence matched the *CP4 EPSPS* sequence of a glyphosate-resistant variety of soybean (GenBank accession AF464188.1).

Based on the original 2003 data, additional searches conducted in 2004–2005 were focused on nonagronomic mesic habitats within a 4.8 km band outside the control area (Reichman et al. 2006; Watrud et al. 2004). These surveys located 55 *Agrostis* ssp. populations on publicly accessible lands, and 34 sites were newly located since the 2003 survey. Nine *CP4 EPSPS* positive plants were identified out of 20,400 tissue samples screened via Traitchek kits, eight of which were found within the new population sites. The presence of the transgene was confirmed in each plant via PCR amplification and sequencing of the PCR product, which again matched that GenBank accessions AF464188.1—*Glycine max CP4 EPSPS* (glyphosate-resistant soybean variety). To establish the parentage of each plant, sophisticated species-level molecular phylogenetic trees were constructed via sequencing of a nuclear encoded ribosomal DNA [internal transcribed spacer (ITS)] and maternally inherited chloroplast-encoded DNA (*matK*). The distribution and phylogenetic information suggested that six of the *CP4 EPSPS* positive plants resulted from pollen-mediated gene flow from the production fields to wild creeping bentgrass plants, while three arose from dispersed seeds (Reichman et al. 2006). The hybridization data with both sentinel plants of creeping bentgrass and resident *Agrostis* spp plants indicate that GE glyphosate resistance in creeping bentgrass can be transmitted to compatible wild relatives at landscape level over multi-kilometer distances. It was estimated that exposure to the *CP4 EPSPS* gene occurred over a total area of over 300 km^2 as a result of the initial year of flowering of the eight GE fields in 2003 (Reichman et al. 2006; Watrud et al. 2004). A significant caveat is that the nine wild transgenic plants above were located in publicly accessible areas limited to roughly 10% of the total estimated *Agrostis* habitat; thus, the surveys may have underestimated the establishment of wild transgenics in the study region (Reichman et al. 2006).

Surveys conducted within the control area from 2003 to 2006 found gene flow within the control area perimeter, as well as gene flow via seed to the northeast from a documented wind event (Mallory-Smith and Zapiola 2008; Zapiola et al. 2008). Glyphosate-resistant plants were identified in situ via TraitChek RUR^{TM} strips (http://www.sdix.com/). Approximately 80 km of irrigation canals, roadsides, ditch and pond banks, and pipelines in the area roughly 300 m around the production fields were surveyed in 2003. While not all the survey sites were necessarily revisited, the surveyed area was increased and extended up to a 5 km radius outside the control perimeter in 2005 and 2006. Of the 57 plants located and tested in 2003, none were herbicide resistant; however, 0.376% of the seeds collected gave rise to glyphosate-resistant seedlings in the greenhouse and had therefore received the *CP4 EPSPS* gene via pollen. In 2006, 3 years after the original GlyRCB fields were taken out of production, 62% of 585 creeping bentgrass plants tested were glyphosate resistant. Strikingly, 0.012% of 49,351 seedlings grown from seed of glyphosate-sensitive plants collected in 2006 were glyphosate resistant, thereby demonstrating that pollen-mediated transgene flow was still occurring despite intensive mitigation efforts by The Scotts Company (http://www.scotts.com/) to totally remove glyphosate-resistant plants from the area (Zapiola et al. 2007). Interestingly, two modeling studies, one based on predictions of creeping bentgrass pollen dispersal based on wind data at the time that the GE creeping bentgrass fields were growing in central Oregon in 2003 (Van de Water et al. 2007), and the other, based on counts of non-GE creeping bentgrass pollen collected near flowering fields with air-samplers in western Oregon (Pfender et al. 2007), each came up with very similar multi-kilometer distances that closely matched the maximal 21 km distance reported for live GE creeping bentgrass pollen that was based on production of F_1 seedlings tested in a greenhouse setting (Watrud et al. 2004).

10.4 Gene Flow via Seed Scatter

Seed scatter is defined as the loss of seed at any time from the beginning of production through final end use. Among perennial grasses, the possible risk of gene flow through seed scatter is high because of seed size, the potential for survival in the seed bank, and for some species subsequent vegetative reproduction. The seeds of most turf and forage grass species are much smaller than those of annual crops and therefore are very difficult to contain during production, collection, and distribution for sale. For instance, creeping bentgrass seeds are approximately 2 mm $\times$ 0.5 mm and may weigh as little as 80 μg each (Reichman et al. 2006). Also, seed viability is much longer than that of pollen. Unlike pollen, there is no "time window" for seed movement—seed movement can happen at many times (e.g., at planting, during or after harvest) and seedbanks can renew gene flow in subsequent years. Furthermore, seed does not require a sexually compatible relative to contribute to gene flow; thus, there is no need for outcrossing to compatible wild relatives.

Because of its small size, perennial grass seed can move easily via natural dispersal vectors, production practices, and in end use, e.g., in golf courses, landscapes, pastures, and forage production. Seed can be dispersed via natural dispersal vectors such as wind, water, and animals—factors over which humans have little to no control. Furthermore, perennial grass seed production involves the movement of seeding, application, and harvesting equipment, as well as seed cleaning, field irrigation and seed distribution via long-distance trucking. Thus, equipment is frequently moved in and out of the field during seed production, increasing the probability of seed escape. Ultimately, the purpose of large-scale seed production is the distribution of the product to customers who are separated by long distances. While seed scatter may be reduced in any one of these steps it cannot be entirely prevented. Unfortunately, there is a paucity of studies that address gene flow through seed scatter. This is probably because most studies to date have been concerned with annual species that generally do not survive outside of cultivation and have little or no seed dormancy. The few exceptions are gene flow studies in canola and sugar beets, neither of which are perennials. Therefore, gene flow via seed scatter presents a serious challenge to gene confinement efforts.

10.4.1 Gene Flow via Seed Escape in Glyphosate-Resistant Bentgrass

In August 2003, after swathing but before threshing, a documented strong northwesterly wind event in the production area moved creeping bentgrass seed and panicles from swathed windrows of the northernmost GlyRCB production field (Zapiola et al. 2008). Mitigation procedures were undertaken, including herbicide treatment and hand rogueing of the field, which substantially reduced the level of GlyRCB volunteers.

Additional surveys were conducted in 2004, 2005, and 2006, both within the control area and to a 5 km radius outside its perimeter. By 2004, glyphosate-resistant plants were found distributed throughout the control area along canals and irrigation ditches, often in places where they were not located in 2003 (Zapiola et al. 2008). The distances of distribution varied from adjacent to a creeping bentgrass production field to 1.9 km from the original closest production field. A total of 300 plants were tested via Traitchek RUR^{TM} strips, 49% of which were identified visually as creeping bentgrass, and 93% of these were *CP4 EPSPS* positive. In 2005, a total of 1,290 plants were tested, with 75% identified as creeping bentgrass, 19.3% redtop, 0.5% rabbitfoot grass, and the remaining 5.2% represented by *Agrostis* ssp. and potential hybrids. Of the total plants tested, 40.5% (522/1290) were glyphosate-resistant, of the creeping bentgrass plants tested, 54% (521/968) were glyphosate-resistant, and the most distant resistant plant was 4.6 km from the nearest original GlyRCB production field. By 2006, 62% of creeping bentgrass plants tested were glyphosate-resistant and the most distant GlyRCB plant was also found 4.6 km away from the nearest original GlyRCB field (Zapiola et al. 2008).

10.5 Future Impacts of Gene Flow from Glyphosate-Resistant Creeping Bentgrass

Although gene flow via pollen dispersal and seed escape occurred during seed production in 2003 and 2004, its impact in future years is still undetermined. The results of this field trial are of public and commercial interest and have significant potential regulatory and policy implications. To date, studies have measured only environmental exposure to GlyRCB, not the long-term effects of gene flow. Numerous unresolved concerns remain; creeping bentgrass seed can remain viable in seed banks for as long as 4 years (C.M.-S., unpublished data), thus its possible contribution to volunteering is uncertain; the potential for contamination of neighboring farms during GlyRCB production could create marketing issues; and the potential for establishment of hybrids and introgression of the glyphosate-tolerance trait into wild populations is uncertain. Further, contamination of irrigation ditches and drainages with herbicide-tolerant grass could make control more difficult and expensive, because glyphosate is one of a few herbicides labeled for use along waterways. It is also not known whether seed can remain viable and move through the irrigation canal system, or how much seed a volunteer plant can produce with no outside pollen sources once the creeping bentgrass fields were removed from production.

On the other hand, persistence of the glyphosate resistant trait in populations of compatible wild relatives without the selective pressure of herbicide is an open question, as the glyphosate resistant trait has not been shown to have a fitness cost (Fei and Nelson 2004). Even with safeguards in place, gene flow via seed and pollen was not contained during the 2002 and 2003 plantings as a natural dispersal mechanism (wind) coupled with hygienic production practices still led to measurable gene flow. GlyRCB plants were found in other crop fields and non-production areas that required increased control measures. Thus, while GlyRCB release into the environment will probably have little environmental impact on wild species per se, it could significantly increase the weed control costs for management of various agronomic and non-agronomic environments. Therefore, the continued development of GlyRCB requires an effective mitigation plan in place that incorporates control measures for all possible sites—crop fields, canals, ditches, non-crop fields, and non-crop areas.

10.6 Conclusions

How have our experience, data, and knowledge about gene flow with regards to regulating food, feed and fiber crops prepared us for the world of dedicated GE biofuels and biomass crops? Furthermore, how much will the creeping bentgrass story impact the future commercialization of other perennial GE grasses such as switchgrass (*Panicum virgatum*)? There are several perspectives pertinent to the future commercialization of a bioenergy feedstock such as switchgrass. These

include: (1) the impacts of regulatory requirements on small scale and prospective corporate developers of GE perennial grasses for bioenergy; (2) the large potential land area for commercial production of a dedicated energy crop such as switchgrass; (3) development of effective biocontainment biotechnologies; and, (4) perceived economic, agronomic, and ecological benefits of engineered perennial grasses for use in bioenergy production.

The deregulation of transgenic plants worldwide has become increasingly more conservative and stringent in recent years, typically focusing on modes of gene transfer (i.e., transgenics), rather than phenotype. The new rules proposed by the USDA-APHIS-BRS (Biotechnology Regulatory Services) (under public comment until June 2009; the agency has said nothing further since 2009 nor issued new rules) are consistent with this trend, and would likely increase the amount of paperwork required permits allowing release into the environment, and thus require more overseeing, even for relatively environmentally benign traits and crops. Therefore, the costs of deregulating a GE plant will likely increase in the future. At the same time, large international agricultural companies are not the primary investors of research funds into the biotechnology of dedicated bioenergy crops. Rather, bioenergy investors are relatively inexperienced companies regarding deregulation—i.e., more like Scotts and less like Monsanto. So, we should expect regulators to take a very long and careful look at perennial bioenergy grasses. On the other hand, two of us (A.P.K. and C.N.S.) have pending BRS permits for releasing transgenic switchgrass into the environment, which will be the first such occurrences. There seem to be few special stipulations with regards to growth requirements, but these will both be very small trials (20 plants).

Second, the scope of potential area under commercialization of switchgrass is huge compared with a golf course grass such as creeping bentgrass. In addition, switchgrass grows over 2 m tall, which is much larger than creeping bentgrass. The potential pollen and seed production of switchgrass relative to bentgrass could translate to high levels of potential gene flow via wind and other vectors. Also, switchgrass is native across much of North America and wild populations would likely be proximate to transgenic populations. So, if unmitigated transgene movement from bentgrass into wild and non-transgenic crop varieties was undesirable, switchgrass would likely be appreciably more challenging. Pollen-mediated gene flow studies in transgenic switchgrass will provide valuable data concerning the need for gene confinement in genetically modified varieties with biofuels-specific traits.

This brings us to the third issue: the necessity for biocontainment in switchgrass, especially to limit gene flow via pollen (Stewart 2007). Fortunately, tools, such as gene deletor technologies based on site-specific recombination (Luo et al. 2007), male sterility and transplastomics (Daniell 2002) exist, and novel tools are under development (H.S. Moon, J.M. Abercrombie, A.P.K., and C.N.S.Jr., unpublished). Unfortunately, none of these seem to be ready for commercialization or have even been tested in perennial feedstock grasses such as switchgrass.

All these issues lead us to exercise caution, albeit optimistic caution, with regards to future commercialization of transgenic switchgrass or other perennial

grasses such as *Miscanthus* (Stewart 2007). Biocontainment strategies should be allowed to co-mature and co-develop with traits of interest, such as domestication traits and cell wall traits for decreased recalcitrance for digestion. While cellulosic bioenergy is certainly a compelling new industry, it must play by the well-established regulatory rules. We have learned enough to know that a mature and regulated bioenergy industry will not occur quickly if it is to be sustainable.

Disclaimer: Mention of trade names does not imply endorsement of the commercial products that are mentioned nor do the views expressed herein necessarily reflect the views of USDA or USEPA.

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