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FORUM

Sustainable Use of Biotechnology for Bioenergy Feedstocks

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Abstract Done correctly, cellulosic bioenergy should be both environmentally and economically beneficial. Carbon sequestration and decreased fossil fuel use are both worthy goals in developing next-generation biofuels. We believe that biotechnology will be needed to significantly improve yield and digestibility of dedicated perennial herbaceous biomass feedstocks, such as switchgrass and *Miscanthus*, which are native to the US and China, respectively. This Forum discusses the sustainability of herbaceous feedstocks relative to the regulation of biotechnology with regards to likely genetically engineered traits. The Forum focuses on two prominent countries wishing to develop their bioeconomies: the US and China. These two countries also share a political desire and regulatory frameworks to enable the commercialization and wide release of transgenic feedstocks with appropriate and safe new genetics. In recent years, regulators in both countries perform regular inspections of transgenic field releases and seriously consider compliance issues, even though the US framework is considered to be more mature and stringent. Transgene flow continues to be a pertinent environmental and regulatory issue with regards to transgenic plants. This concern is largely driven by consumer issues and ecological uncertainties. Regulators are concerned about large-scale releases of transgenic crops that have sexually compatible crops or wild relatives that can stably harbor transgenes via hybridization and introgression. Therefore, prior to the commercialization or extensive field testing of transgenic bioenergy feedstocks, we recommend that mechanisms that ensure biocontainment of transgenes be instituted, especially for perennial grasses. A cautionary case study will be presented in which a plant's biology and ecology conspired against regulatory constraints in a non-biomass crop perennial grass (creeping bentgrass, *Agrostis stolonifera*), in which biocontainment was not attained. Appropriate technologies that could be applied to perennial grass feedstocks for biocontainment are discussed.

Keywords Biofuels · Switchgrass · Miscanthus · China · Biocontainment · Sustainability · Regulations

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Introduction

Why Biotechnology is Needed for Biofuel Feedstocks

If bioenergy platforms such as switchgrass-to-cellulosic ethanol are to be economically viable, improved feedstocks must be developed that have high yield and decreased recalcitrance for the conversion of cell walls to fermentable sugars. To attain these goals, biotechnology will likely be needed to make improvements in feedstock (Gressel 2008). Several feedstock candidates, such as *Panicum virgatum* (switchgrass) have a number of wild traits and are not very domesticated compared with current row crops. This being the case, rapid gains should be attainable with plant breeding, especially for traits that are based on endogenous genetic variation. These traits include yield and dwarfism, which were foundational to the Green Revolution (Fernandez and others 2009). In contrast, other traits, such those related to recalcitrance, improved processing, and introduced bioproducts might be more readily conferred by biotechnology (Jacob and others 2009; Sainz 2009). In another example where biotechnology could be revolutionary, switchgrass or other C4 grasses could receive a large biomass boost from adding a single *Miscanthus* gene to increase cold temperature photosynthesis. The putative mechanism by which *Miscanthus* × *giganteus* maintains photosynthetic efficiency at cool temperatures is the result of the expression patterns and activity of a single C4-pathway enzyme, pyruvate phosphate dikinase (PPDK) (Wang and others 2008; Dohleman and Long 2009). Thus, by simply increasing the expression of the PPDK gene in switchgrass, this crop could conceivably add significant biomass at early and late stages of the plant's growing season (Stewart and others, unpublished).

Regulatory costs and concerns are important considerations that must be made when transgenic plants are released into the environment, especially for commercialization. Both process and product of transgenic plants is regulated by most governments throughout the world, including the United States and China. Therefore, one important facet regarding sustainability of growing any transgenic biomass crop, such as switchgrass, is of a regulatory nature. Therefore, when we refer to sustainability in this paper—in the context of releasing transgenic plants—we explicitly consider that there should be an absence of negative environmental or regulatory events directly associated with transgenic plants. This absence is required for them to be usable over a number of years or decades.

Regulators make decisions using risk assessment, which, in general, and specifically for biotechnology, is well developed (see Wolt and others 2009).We realize that sustainability is a complex concept that is not usually applied with regards to the regulatory durability of plants derived from biotechnology, but we would like to propose that promise of regulatory-driven sustainability is a prerequisite for the release of any transgenic plants. First, without the reasonable assurance of sustained compliance to regulations, a company will likely not invest funds to protect and implement the intellectual property required in biotechnology. Secondly, and related, biotechnology would likely not be deployed if there were a reasonable chance that a transgenic plant would be deemed environmentally hazardous and not approved by regulatory officials.

Two important aspects of regulation and biosafety will be addressed here, and additional regulatory and risk assessment issues of bioenergy plants are discussed elsewhere (Wolt 2009). First, increasing yield, stress tolerance and other traits could conceivably also increase invasiveness or weediness of the transgenic crop itself (Warwick and Stewart 2005). Thus, whether introduced via breeding or biotechnology, new traits must be analyzed *a priori* for their potential to increase invasiveness, and then, critical field experiments must be performed prior to commercialization (e.g., Halfhill and others 2005). Second, gene flow from transgenic feedstocks to non-transgenic relatives, either crop or wild (Stewart and others 2003), must be prevented or mitigated (Stewart 2007; Kausch and others 2010). In this Forum, we give an overview of several biotechnology tools that could be useful for controlling transgene flow in perennial biomass grasses. Prevention of transgene flow is especially important when species or genera are indigenous to the region of intended cultivation, such as switchgrass in the US and *Miscanthus* in China (Stewart and others 2003; Stewart 2007).

Transgenic crops have been grown commercially for 15 years, but no transgenic dedicated bioenergy feedstocks have yet to be commercialized. It will be important to rationally design transgenic feedstocks for environmental and regulatory-driven sustainability, as well as for bioenergy goals. It is doubtful we will get a second chance to get these things right if we get them wrong the first time. It is interesting to note that environmental sustainability is a major driver for the creation of the new bioeconomy. Everyone agrees that petroleum will eventually run out, and even if that were not the case, there are dire concerns over carbon emissions. Energy derived from perennial herbaceous grass biomass promises to actually sequester more carbon than emitted (Yuan and others 2008). Thus, if biotechnology can improve a plant so that it produces more biomass without invasiveness or compromising ecological functions, then environmental sustainability of a new industry can be facilitated by biotechnology. One large impediment to this realization is that biotechnology is specifically regulated as a mode to plant improvement. In some extreme cases, transgenic plants are totally banned from being grown in some regions, especially in Europe.

The Role of Biotechnology in Bioenergy

Globally, bioenergy demands are diverse and affected by the specific environmental constraints of a given region (Wright 1994). In this context, it becomes clear that there may be many paths and solutions towards a sustainable energy future (Yuan and others 2008). The paths consist of potential various crops, land use, process technologies, and products. We are in the midst of an exciting transition in the field of bioenergy that includes scientific and engineering innovations toward the end of an emerging bioeconomy.

There has been a surging interest in optimizing the ability to extract fermentable sugars from plant-derived cellulose, earth's most abundant energy-rich polymer, for the production of ethanol and other liquid fuels (Miller and Keller 2009). The challenges inherent in this process involve complex biological and chemical problems that must be addressed to develop feasible infrastructure and efficient processes for cellulosic ethanol production from biomass. These problems range from understanding plant cell wall biology to addressing the chemical recalcitrance of biomass conversion (Miller and Keller 2009). Increased knowledge in basic science and technology should result in more effective strategies to achieve goals of sustainability. Instead of exploring the many biological approaches that plant genetic engineers are currently pursuing towards meeting these goals, we focus our discussion here on possible solutions to plant gene flow challenges that must be addressed in order for regulatory-driven sustainability of feedstock biotechnology to be a reality. While we will emphasize switchgrass in the US, the information is applicable to other bioenergy crops, especially dedicated herbaceous feedstocks in the US and native feedstocks in other countries, especially *Miscanthus* in China. Indeed, it is crucial that China be discussed, since it has the fastest growing fossil fuel consumption rate in the world paired with commensurate opportunities and challenges in the development of bioenergy solutions (Wang and others 2009).

Why Switchgrass?

Several plant species and plant-to-fuel platforms, including switchgrass to cellulosic ethanol are typically discussed as biofuel solutions (Yuan and others 2008). Switchgrass (*Panicum virgatum* L.) is a perennial warm-season forage grass indigenous to North America. In the 1990s, there was some progress to determine the adaptability of various potential dedicated biomass crops to regions in the US (Wright 1994), although biotechnology was not considered as a factor. In the Wright analysis, switchgrass played a prominent role as a plant adapted to many regions of the US. Furthermore, it does not seem to be invasive. Originally adopted as a forage crop (Parrish and Fike 2005), it is now considered to be one of the most promising emerging cellulosic biomass crops. In 1992, the US Department of Energy started a research program focused on developing switchgrass as a sustainable bioenergy feedstock (Sanderson and others 1996). Switchgrass was chosen as a biomass-based renewable energy source crop because it had high forage yield and seed production at different regional cultivar testing fields in several states in the US (Sanderson and others 1996). Switchgrass cv. Alamo has been ranked as the highest yielding cultivar in most yield trials conducted and is relatively amenable to genetic transformation and subsequent regeneration into mature plants (Sanderson and others 1996; McLaughlin and Kszos 2005; Burris and others 2009). Unlike maize starch, the major source of ethanol in the US, switchgrass production should not directly affect food prices since it is not used for human consumption. Additionally, switchgrass can be grown and cultivated on marginal lands, thereby alleviating concerns about biofuel crops directly competing with food crops. The best-case land-use scenario for switchgrass is its cultivation on agriculturally-depleted soils that no longer support agriculturally important row crops.

Environmental Concerns with Transgenic Switchgrass

Biotechnology can be used to study and improve biosynthesis traits and biomass, which, in turn should increase cellulosic ethanol yield. The potential for harnessing biotechnology for feedstock improvement has been recently reviewed (Yuan and others 2008). In addition to the environmental concerns about the cultivation of both food crops and feedstock crops for bioenergy that range from land use to carbon footprints, additional environmental and regulatory concerns revolve around potential transgene escape from transgenic switchgrass populations to non-transgenic switchgrass, and potentially also to wild relatives (Palmer 1974; Stewart 2007). Non-transgenic switchgrass that could be deemed at-risk might be used for livestock feed, fuel, or they could simply be wild-growing plants. Transgenic traits engineered into switchgrass for improved ethanol production might not be suitable as a forage crop for livestock feed and could have negative consequences in free-living populations. For example, introducing a dwarfing gene for decreased lodging or modification of lignin biosynthesis pathways could have deleterious effects on non-transgenic populations by making them less competitive. Since switchgrass is known as a wind-pollinated obligate-outcrosser, large-scale farming of transgenic switchgrass could provide many opportunities for transgene escape into wild populations. To date, there are no regulatory-driven studies (i.e., with transgene escape in mind) of gene flow and environmental consequences of transgenic switchgrass, so regulators will likely take a very cautious approach when developing policy (Stewart 2007). For example, in 2009, the USDA-APHIS-BRS approved the first transgenic switchgrass for regulated release into the environment. As a condition for issuing the permit, we (and others) were required to not allow plants to flower, shed pollen, and set seeds. Given the current regulatory landscape in the US, we believe that transgene containment strategies must be in place prior to commercialization of transgenic switchgrass. Transgene containment will help ensure that the use of transgenic approaches for genetic improvement of switchgrass will be sustainable from a regulatory perspective; acceptable level of environmental risk with regards to gene flow must be attained.

There has been a plethora of research on gene flow from transgenic plants (reviewed in Stewart and others 2003), but relatively little is known about transgene flow in perennial grasses. Understanding the potential for transgene escape and its theoretical environmental consequences is particularly important when considering obligate outcrossers like switchgrass. Transgene escape from transgenic plants to either sexually compatible non-transgenic plant populations or wild relatives has been demonstrated under natural field conditions in *Brassica napus* (canola), a facultative outcrossing dicot (Warwick and others 2003; Warwick and others 2008). Transgenes can flow from transgenic plant populations to non-transgenic plants through vegetative propagules (e.g. rhizomes and stolons in some grasses), seed dispersal, or dispersal of pollen by wind, insect, or other vectors (Llewellyn and Fitt 1996). However, most transgene flow from cultivated transgenic crops occurs by cross-pollination (Lu 2003; Stewart and others 2003). This mechanism of transgene dispersal requires overlapping flowering periods between transgenic donor plants and non-transgenic recipient plant populations (Simard and Légère 2004; Légère 2005). Transgene escape has been reported from transgenic varieties to cultivated non-transgenic varieties in rice and canola under agronomic field conditions (Messeguer and others 2001; Rieger and others 2001). The potential for hybridization and transgene introgression of crop plants such as wheat, sunflower, rice, sorghum and canola with their closely related wild relatives in a field setting is also one of the major concerns (Ellstrand and others 1999; Halfhill and others 2004). Despite the potential for sorghum as a bioenergy feedstock, this crop would be under very close scrutiny as a candidate for genetic manipulation and commercial release because of the high risk of transgene introgression through hybridization with wild Johnsongrass, which is one of the most notorious weeds on US. cropland (Stewart and others 2003; Clements and others 2004). It is uncertain how many sexually-compatible *Panicum* species exist that could hybridize with switchgrass. *Panicum* is a large genus in North America with over 150 species, so, on the surface, there could be gene flow issues for switchgrass to wild relatives, especially with the Virgatum group of six species (Palmer 1974). While a few *Panicum* species can be agricultural weeds, none approach the noxiousness of Johnsongrass, a weedy relative of sorghum.

Public perception and acceptance will also play important roles in the successful adoption of transgenic energy crops (Stewart 2007). Public mistrust about transgenic plants was fueled when transgene escape was reported in experimental plots of *Agrostis stolonifera* (creeping bentgrass). The plants in question were engineered for tolerance to the popular RoundUp® (glyphosate) herbicide. Herbicide-tolerant creeping bentgrass was the first transgenic perennial grass that was intended to be taken through the US regulatory process toward commercialization. In a preliminary risk assessment, the USDA-APHIS-BRS determined that the glyphosate-tolerant creeping bentgrass line was not significantly different from its parental line except for its tolerance to the herbicide. It was also determined that it was not sexually compatible with any threatened, endangered, or noxious plant species. Therefore, APHIS-BRS approved a 162 ha experimental planting to assess and monitor gene flow to conspecific bentgrass prior to commercial release. Unfortunately for the USDA and the commercial proponent, this experimental plot had negative consequences to commercial producers of grass seed in the Willamette Valley of western Oregon, where 70% of the US grass seed is produced. Creeping bentgrass seeds are very small ($\sim$13,500 seeds g^{-1}) and it is a wind-pollinated, highly outcrossing, perennial grass. Turfgrass breeders and growers in Oregon were quite concerned that genes would flow to non-transgenic creeping bentgrass breeding and production fields, that there would be the potential generation of RoundUp®-resistant weeds and increase in cost of new herbicide control of these weeds, and that they also questioned who would incur those costs if contamination occurred (Zapiola and others 2008; Kausch and others 2010). In the end, all these concerns were validated. There was extensive gene flow of the herbicide-resistance gene; found up to 21 km away from source plants (Watrud and others 2004; Zapiola and others 2008). In the fallout of this debacle, the USDA was successfully sued and transgenic bentgrass commercialization was placed in direct jeopardy. In another instance, the USDA was also sued over field releases of herbicide resistant alfalfa—again a gene flow issue; this case appeared before US Supreme Court in April 2010 (at the time of submission, a decision had not been reached). In both of these cases free-living non-transgenic plants of the same species are found in the same areas of cultivation. In yet another example, in 2006 Anheuser-Busch (St. Louis, Missouri, USA) objected to Ventria Bioscience's (Sacramento, California, USA) pharmaceutical-expressing transgenic rice being cultivated near the beer company's rice production fields because of worries of adventitious transgene presence (Stewart 2008). Gene flow has proven to be a critical issue surrounding the regulation of transgenic plants, thus begging for containment technologies (Stewart 2007, 2008; Kausch and others 2010).

There have been no studies reported on transgene flow of switchgrass. A study of genetically modified tall fescue (*Festuca arundinacea* Schreb.) has shown that the maximum effective travel distance of transgenic pollen was 150 m from the donor plants (Wang and others 2004). However, it has been shown that the maximum distance of cross-pollination for maize pollen is 200 m from the pollen source plot (Luna and others 2001). The average size of a corn pollen grain is 91–93 µm in diameter, while the size of switchgrass pollen is about 40 µm in diameter (Aylor

2002; Richards and others 2001). Because of the smaller size of switchgrass pollen, it might be expected to travel further distances by wind than corn pollen. Of course, there are many factors that could contribute to gene flow via pollen, and these should be the subject of future research.

Transgene Biocontainment Strategies for Sustainable Use of Biotechnology

Biocontainment of feedstock transgenes has been reviewed recently (Kausch and others 2010). Transgene containment strategies are desirable to suppress unintentional transgene escape from transgenic plant populations to sexually compatible non-transgenic plant populations. Physical transgene containment strategies include building fences or planting border plants as mechanical barriers around transgenic plant populations. Another physical method is the removal of flowers prior to anthesis. Physical containment generally has very limited capability for pollen flow control. For example, after mid-summer, switchgrass flowers continually, requiring removal of flowers once per week or more frequently.

Many of the more sophisticated biological transgene containment (biocontainment) strategies have exhibited high efficiency in controlling transgene movement via pollen dispersal, at least in laboratory or greenhouse settings, where mechanisms have generally been studied. In addition, these biocontainment strategies have been mostly demonstrated in model plants, and not in crops or bioenergy feedstocks. Currently, several biological strategies of transgene containment, such as male sterility, maternal inheritance, transgene mitigation, and transgene excision, have been shown to be useful in controlling transgene escape (reviewed in Daniell 2002; Luo and others 2007; Gressel 2008; Kausch and others 2010).

Male sterility has been used extensively in commercial agriculture for the production of F_1 hybrids. In the reproductive cycles of higher plants, viable pollen is required for successful pollen germination, pollen tube growth, and eventual double-fertilization via transmission of the sperm cells to the ovule. Disrupting pollen development through genetic manipulation has been used for suppressing transgene escape and flow. For example, many male-sterile plants have been genetically engineered using constructs that disrupt the tapetum, a layer of cells found within the pollen sac that is essential for pollen development (reviewed in Daniell 2002). Male sterility has been achieved using tapetum-specific promoters to drive the expression of a toxic bacterial genes (e.g., *Barnase* from *Bacillus amyliquefaciens* or diphtheria toxin A), which results in pollen ablation (Hird and others 1993; Koltunow and others 1990; Mariani and others 1990). Expression of the chimeric ribonuclease genes prevented pollen formation and resulted in male sterility that could be used as a transgene containment strategy in bioenergy crops. The only commercial result of this technology has been the *Barnase Barstar* system, which enabled transgenic canola to be cultivated in Canada and Europe. Recent researchers have focused on using genetic engineering strategies that utilize endogenous plant genes to achieve male sterility with some success in *Arabidopsis* (Konagaya and others 2008). Plant genes for male sterility could be received more favorably by the public compared with, say, diphtheria toxin genes. As plant genomic resources expand, similar engineering strategies could be feasible using endogenous genes from the host plant (e.g., switchgrass) or another closely-related species.

Cytoplasm-based strategies represent possible options for gene flow biocontainment. Cytoplasmic male sterility (CMS) can be exploited for transgene containment by blocking the production of functional pollen derived from mutations in the plant mitochondrial genome (Hanson and Bentolila 2004). One potential issue to consider is that fertility of CMS plants can be easily restored when appropriate nuclear genes are introduced that overcome CMS (Chase 2006). Also the loss of fertility in a CMS breeding plant population can be eventually restored under natural conditions (Schnable and Wise 1994).

Plastid transformation shows potential for circumventing transgene flow via pollen. Maternally-inherited plant plastid genomes in most crop species provide several advantages, such as high level of transgene expression and expression of multiple operons in the genome (Maliga 2004). Since plastids are not maternally inherited in all plant species, the use of plastid-based male sterility may be limited to some plant species (Hagemann 2004). Maternal inheritance of transgenes in plastids of transgenic plants and suppression of transgene dispersal through pollen has been demonstrated in tobacco and tomato (Daniell and others 1998; Ruf and others 2001). Integrating transgenes in tobacco plastid DNA enabled gene containment of transgenic plants via maternal inheritance of their plastids (Iamtham and Day 2000). Transformed chloroplasts of tobacco with herbicide resistance genes were inherited maternally instead of being transmitted via pollen (Daniell and others 1998). Despite its potential for biocontainment, plastid transformation has only been successfully established in limited numbers of plant species and is still a large technical hurdle, especially in the grasses.

A strategy called transgene mitigation could be effective for some bioenergy crops. Linked to a primary gene of interest, a mitigating gene, which is positive or neutral for crops, but negative or deleterious for potential non-transgenic hosts is introduced into the crop (Al-Ahmad and others 2004). Transgene mitigation as a gene containment strategy has been demonstrated in tobacco and canola using a dwarfism gene (Δ *gai*: ***g***ibberellic ***a***cid ***i***nsensitive) that produced less competitive hybrid populations than non-transgenic weed populations (Al-Ahmad and others 2004, 2006; Rose and others 2009). Reproductive fitness of transgenic canola containing the dwarfism and herbicide-resistance genes was significantly less than non-transgenic canola volunteer competitor when selective herbicide was not used (Al-Ahmad and others 2006).

Another strategy for transgene containment utilizes transgene excision by site-specific recombination (reviewed by Moon and others 2010). Site-specific recombination systems allow for the removal of a transgene from the transgenic plant genome prior to pollen dispersal and fertilization, thus blocking the release of the transgene into the environment. Several site-specific recombinases were proven to be efficient for the excision of transgenes from transgenic plant pollen and/or seeds (Luo and others 2007; Moon and others 2010). Researchers are currently developing transgene containment strategies that might be more efficient and fail-safe for employment in a transgenic switchgrass crop (Stewart and others, unpublished). Although transgenic switchgrass cultivation for ethanol production seems to be free of concerns related to human consumption, detailed studies will be required to address possible environmental impacts and related regulatory issues (Wolt 2009). Ultimately, if the development of 100% fail-safe measures is not feasible, then acceptable threshold levels of risk must be determined and policy decisions will likely cautiously follow.

Perspectives

There are many uncertainties about next-generation biofuels, in this case, cellulosic ethanol. Selection of feedstocks, biotechnology improvements, regulatory requirements, feedstock processing and fuel processing must be developed simultaneously and in a complementary fashion; there are many available choices (Yuan and others 2008). We do not know whether consolidated bioprocessing or some other biological or catalytic process will be the best one for breaking down cell walls into sugars. There is much discussion about preprocessing feedstocks for optimal sugar release. As we traverse further upstream to the feedstock, we see that there are also multiple choices, each with benefits and risks (Yuan and others 2008). Nearly all of the choices, including switchgrass, are immature as crops, agronomically, and most do not have current economic uses. Therefore, if we assume that we need to domesticate and solve the recalcitrance problem directly for any feedstock, then biotechnology and/or synthetic biology will likely be employed, which have a number of real and perceived risks. The current regulatory pendulum in the US for agriculture and food biotechnology has swung towards the conservative side. Regulatory-driven sustainability is an absolute requirement for a sustainable biofuels industry using biotechnology, therefore gene flow and other biotechnology risks must be addressed prior to deployment. Given the conservative nature of regulations and regulators, any deregulated transgenic switchgrass or *Miscanthus* will likely be required to have extraordinarily high biosafety. Many uncertainties could conspire to delay next generation biofuels, but if we have learned anything from the GMO era, it is that biosafety must be considered as an integral part of the research and development plan in a regulated environment (Stewart Jr. 2004).

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