REVIEW

Transgenic perennial biofuel feedstocks and strategies for bioconfinement

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The use of transgenic tools for the improvement of plant feedstocks will be required to realize the full economic and environmental benefits of cellulosic and other biofuels, particularly from perennial plants. Traits that are targets for improvement of biofuels crops include herbicide resistance, pest, drought, cold and salt tolerance, nutrient use efficiency, altered cell wall composition and improved processing and end-use characteristics. However, controlling gene flow is a major issue and there is no regulatory experience with perennial plants as dedicated biofuels feedstocks. Bioconfinement of transgenes is thus an obvious regulatory and biosafety objective to the release and commercialization of transgenic bioenergy feedstocks. In this article, we review bioconfinement strategies that target pollen or seeds that can be applied to perennial plants used as biofuels. These include male sterility, integration of transgenes into plastid genomes, removal of transgenes in pollen and seeds, transgene expression in vegetative organs for harvest before appearance of reproductive structures or gene use restriction technologies.

Limitations in the current availability of bioenergy feedstocks are a major problem in next-generation biofuels. There are global economic, political and environmental pressures to increase biofuel production and utilization, to offset gasoline and diesel fuel use, especially in the transportation sector. Many countries, such as the USA and China, have issued increasingly aggressive targets for renewable energy over time; these will certainly require new dedicated feedstocks and fuel platforms [1,2]. Current strategies for liquid fuel production are focused on using ethanol as a gasoline additive and offset, which utilize fermentation of plant-produced starches and sugars, mostly from maize grain and sugar cane, to produce ethanol. It is doubtful whether sufficient amounts of these feedstocks can be supplied without impacting the agricultural sector and harming the environment. It is necessary to develop biofuels produced from dedicated nonfood cellulosic feedstocks that can be produced on land currently unutilized for food production. While most national and international regulatory agencies do not make distinctions based on end use, whether as food, fiber, timber, medicine or any other type of feedstock, the perennial nature of biofuel feedstocks and expediency of current needs requires special attention. In fact, there are several commercially available first-generation transgenic **biofuel feedstocks**, including maize grown for ethanol and canola/rapeseed grown for biodiesel, that are cultivated internationally on a multimillion hectare scale. In both cases, these crops can also be used for food and feed; these are annual crops.

Meeting the goals of the US Department of Energy (DOE) billion ton annual supply of biomass translates into 5% of the nation's power, 20% of the nation's transportation fuels and 25% of the nation's chemicals

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Key terms

Biofuel feedstocks: Plant-derived organic materials used in the production of biofuels such as syngas, biomethane, B100 biodiesel, E100 ethanol and petroleum products

Gene flow: Transfer of alleles of genes from one population to another, resulting in changes in allelic frequencies into or out of a population and responsible for marked changes in proportions of individuals with a particular gene, such as a transgene

Biotechnology: Process that uses the technological applications and modifications of biological systems and their derivatives to make or modify products or processes for specific use to humans and other organisms

by 2030. This goal is equivalent to 30% of current petroleum consumption. The USDA/DOE projects that 42 million acres of cropland will be competitive, producing an average of 4.2 dry tons per acre of perennial grasses at US$40/dry ton [3]. Yields from the best clones of perennial grasses were generally 8 tons per acre or higher and the highest yields of existing clones is 15.5 dry tons per acre. Assuming an intensive genetic and research program, the feasibility of obtaining average yields of 8 dry tons per acre over millions of acres is supported by modeling [4].

The regulatory parameters for maize, soybean, cotton and canola are already well established and **gene flow** studies have been conducted over the last decade. We are now encountering new technologies for the improvement of perennial nonfood plants specifically as feedstocks with biofuel-related traits, which include perennial grasses, such as switchgrass and *Miscanthus,* but also trees: *Eucalyptus*, *Salix, Paulownia, Populus*, *Jatropha*, *Croton* and other biodiesel tree crops. The genetic improvement of food row crops has been greatly accelerated through advanced applications of tools of **biotechnology** and advanced breeding [5] and, undoubtedly, this same model will be useful for improving perennial bioenergy feedstocks [6,7]. The objective of the current review focuses on the challenges to agricultural production of transgenic perennial plants improved for biofuels purposes.

Rapid genetic improvement of the most promising perennial grass feedstocks, such as switchgrass and *Miscanthus,* which are not highly domesticated, are thus anticipated by molecular-assisted breeding methods. In addition, biofuel-specific traits, such as production of glycosyl hydrolases, biopolymers, altered sugar, low-starch or low-lignocellulose fibers and cell wall biosynthesis proteins for increased cellulose and decreased lignin can be engineered to increase fuel production per acre [7–9]. Other major biofuel crop feedstock improvements might be facilitated by transgenic microorganisms for the enhanced fermentation of lignocellulosic biomass to biofuels such as ethanol or hydrogen. The use of biotechnology to improve any feedstock is in its infancy, yet it offers significant potential to improve the utility and production of these cropping systems. In addition, there are also rapidly growing genomics resources for feedstocks. The draft genomes of hybrid poplar [10] and sorghum [11] have been published. The Joint Genome Institute of the US DOE (Walnut Creek, CA, USA) is currently performing shotgun sequencing of the switchgrass genome. There are also several metagenome projects designed to discover new enzymes from cell wall-degrading bacteria and fungi. However, in contrast to the situation for food and fiber crops, it might not be economically feasible to deploy cellulosic feedstocks without addressing both the need to improve the agronomic aspects of their growth on a commercial scale, as well as the recalcitrance problem (i.e., the integrity of the cell walls that makes digestion to simple sugars difficult and costly) in the feedstock itself. Transgenic input traits for traditional row crops have had tremendous economic and environmental benefits, but maize, soybean, cotton and canola were already successfully cultivated in a mature industry prior to biotechnological innovations. By contrast, cellulosic feedstocks have yet to be widely grown and all suffer from the recalcitrance problem. Currently, the cost of pretreatment and exogenous enzymatic digestion to break down cell walls renders cellulosic biofuels uncompetitive with starch-based ethanol [8]. Likely, some combination of transgenes will be needed to address the recalcitrance problem and also to increase current yields and establish sustainability. Owing to this need for a biotechnological approach to both establish feedstock agriculture and to solve processing problems, we believe that perhaps the greatest hurdle standing in the way of the commercialization of transgenic perennial feedstocks and their widescale deployment involves environmental regulation and biosafety.

Although there is an absence of documented risks of gene flow among commercially grown transgenic crops [12], commercial-scale production of certain combinations of transgenic traits and crops could potentially lead to undesirable environmental, commercial trespass and agricultural consequences. This is because many of the traits that are beneficial to the feedstock industry potentially impact plant fitness and the ability of the plants to compete for resources [12,13]. Thus, in all probability, the main biosafety and regulatory issue that will receive immediate scrutiny among transgenic bioenergy feedstocks will revolve around transgene flow from cultivated fields to nontransgenic sexually compatible conspecifics and congeners [14,15]. The main mechanisms for gene flow between transgenic and wild relatives will be via pollen-mediated gene flow, seed scatter and vegetative propagation. Therefore, to realize the full potential of agricultural biotechnology for dedicated energy crop enhancement, the ecological, economic and commercial impacts of gene flow and methods for control must be addressed.

The case of gene flow in creeping bentgrass

Currently, there is extensive research to use biotechnology for bioenergy with the goal of achieving renewable and affordable cellulosic biofuel production. Most of the plants considered as top choices for cellulosic biomass

are perennials that have wild relatives in the areas where they will be commercially produced. While reviewed on a case-by-case basis, bioconfinement of engineered genes in perennial plants used for cellulosic biofuels will, accordingly, be a likely prerequisite for deregulation and commercial production of these plants [14,15]. Creeping bentgrass (*Agrostis stolonifera* L.) was the first transgenic perennial grass to be field tested on a large scale and current information strongly indicates the potential for gene flow in open pollinated transgenic bentgrass to conspecifics [16–22]. This case of a perennial transgene flow through pollen and seeds for creeping bentgrass serves as a cautionary tale for biomass grasses, which are taller and likely to have higher pollen loads.

Depending upon species, gene flow from a transgenic crop can be within or among genetically related species, with the most problematic situation being that of introgression into wild relatives [23]. The frequency of field-level interspecific hybridization by pollen-mediated gene flow was assessed between transgenic herbicide-resistant (*bar* gene) creeping bentgrass (*Agrostis stolonifera* L.) and five related *Agrostis* species: 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) [22]. Two identical transgenic plots were created, which were separated by 140 m, each containing a hexagonal array including 90 sample points for pollen reception and a central point for pollen dispersal. Interspecific hybrids were recovered between transgenic *A. stolonifera, A. castellana* and *A. capillaris* (at frequencies of 0.04 and 0.002%, respectively), but not for *A. canina* and *A. gigantea*. The intraspecific hybridization resulting from pollination with nontransgenic *A. stolonifera* was significantly higher (0.63%). Hybrids were fertile. While these were small-scale field plots, the interspecific hybridization frequencies are valuable for estimating potential exposures prior to potential commercialization (i.e., early in the assessment process).

For creeping bentgrass, perhaps a greater issue is intraspecific gene flow, especially by pollen. In late 2002, under the US Department of Agriculture Animal and Plant Health Inspection Service–Biotechnology Regulatory Services (USDA APHIS–BRS)-regulated status, 162 ha of a glyphosate-tolerant creeping bentgrass was planted within a 4553-ha control area in central Oregon. An additional 2.4-ha field was planted in 2003 that flowered and produced seed in 2004 [18]. This turfgrass variety contained the *EPSPS* gene from *Agrobacterium tumefaciens* strain CP4 and is the first example of a transgenic perennial grass crop that was petitioned to be deregulated by the APHIS–BRS regulatory process. The preliminary risk assessment by BRS concluded that the genetically engineered line used in the study (ASR368) was not significantly different from its parental line except for the transgenic trait of tolerance to glyphosate. In addition, it was deemed to be not sexually compatible with any threatened or endangered species or any species on the Federal Noxious Weed List.

Creeping bentgrass was chosen as a potential commercial transgenic release on golf courses because of its desirable wear and stand qualities. The herbicide tolerance trait was expected to enable easier and better weed control on golf courses. It was expected that commercial golf course-grown creeping bentgrass would not go to flower and seed because of intense mowing practices. However, the field-release plots were not mowed and were allowed to flower and produce seed. 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 release raised concerns among many commercial grass seed producers in the Willamette Valley of Western Oregon, where 70% of US grass seed is produced. Creeping bentgrass is self-incompatible and wind pollinated. It can hybridize with compatible species and reproduce by vegetative stolons that can persist and propagate outside of cultivation. Creeping bentgrass seeds are extremely small, approximately 13,500 seeds g^{-1} [24]. Therefore, grass seed producers were concerned about the potential for gene flow to nontransgenic creeping bentgrass seed production and breeding fields. In response to these concerns, a 4553-ha control district was established by the Oregon Department of Agriculture (ODA) in Jefferson County Oregon. This control district was intentionally located more than 150 km east of Oregon's Willamette Valley grass seed production area with some of the following requirements [18]:

- Nontransgenic *Agrostis* spp. could not be grown, planted or handled within the control district;
- Field borders, ditch banks and roadsides within 50 m of the transgenic plots were to be kept free of *Agrostis* spp.;
- Transgenic fields were located more than 400 m away from any creeping bentgrass field outside the control district.

Additional safeguards implemented to prevent unintended seed movement included relatively standard BRS requirements, such as transport of seed in sealed containers to and from fields, cleaning of equipment prior to leaving the field, use of dedicated combines for the GM crop, burning of straw remaining in the field to destroy any seed left behind, and cleaning and

Key term

Transgenic plants: Plants that contain a stable integtration of a gene or genes and/or regulatory elements that have been transferred from a different species or genotype

packaging of seed produced in the control district within the same area. Thus, several specific precautions were required to be taken by the transgenic creeping bentgrass seed producers to help prevent seed scatter from the RoundUp® Ready production fields during this experimental field release, which was regulated under federal USDA–APHIS and state ODA regulatory requirements.

▪ Gene flow by pollen in bentgrass

Bentgrass biology should predicate caution with regards to gene flow and commercial release. It is a perennial grass with more than 30 species of *Agrostis* that occur in North America; approximately 12 species are found in Oregon [201]. In contrast, all current commercial transgenic crops are annuals with few-to-no wild relatives [25]. Creeping bentgrass (*A. stolonifera* L.), 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) can form a hybridizing complex of interpollinating, cross-compatible species. Most *Agrostis* hybrids are sterile or have reduced fertility; given their perennial nature, partial fertility can result in transgene persistence through bridge crosses and possible introgression [22,26].

In 2003, gene flow was monitored outside the 4553-ha control district using nontransgenic endogenous (69 plants) and planted (178 plants) bentgrass, which acted as potential transgenic pollen recipients [19,20]. Seeds were collected from sentinel plants and then germinated and screened for resistance to glyphosate in the greenhouse. Multiple pollen-mediated hybridization events occurred at several kilometers from the transgenic fields; these distances were much longer than reported earlier. The *CP4 EPSPS* transgene was found in seedlings recovered from resident *A. stolonifera* and *A. gigantea* and also in sentinel *A. stolonifera* at maximal distances of 8, 14 and 21 km, respectively. A total of 75 resistant seedlings were collected from 138 *A. stolonifera* sentinel plants, with a 2.0% long-range hybridization rate (625 positive of 32,000 total seedlings tested). A transgenic hybridization frequency of 0.04% (159 positive out of 397,000 seedlings tested) was observed in seeds collected from wild resident *A. gigantea*.

After the 2003 findings, the search for long-range hybridization events was expanded to nonagronomic mesic habitats within a 4.8-km band outside the control area [19,20]. These surveys located 55 *Agrostis* ssp. populations on publicly accessible lands, which yielded nine transgenic glyphosate-resistant plants of 20,400 tissue samples that were tested. In 2006, 3 years after the original transgenic bentgrass fields were taken out of production, 62% of 585 creeping bentgrass plants tested were glyphosate tolerant. Strikingly, 0.012% of 49,351 seedlings grown from seed of glyphosate-sensitive plants collected in 2006 were glyphosate tolerant, thereby demonstrating that pollen-mediated transgene flow was still occurring, despite intensive mitigation efforts by The Scotts Company to totally remove glyphosate-tolerant plants from the area [17].

▪ Gene flow by seed scatter

Seed scatter is potentially problematic among perennial grasses because of small seed size, seed banking and subsequent vegetative reproduction. The seed of most turf and forage grass species is much smaller and lighter than that of annual crops and therefore are very difficult to contain during production, collection and distribution for sale. For instance, bentgrass seeds are approximately 2 × 0.5 mm and weigh as little as 80 µg each [19]. Also, seed viability is much longer than that of pollen; seeds can be scattered at many steps during production (e.g., at planting and 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. Seed planting and harvesting equipment can be moved between fields and, if not thoroughly cleaned, can contain transgenic seeds. Grass seed may also be shipped long distances by road, rail, sea and air, from production to distribution centers and to end-user fields. Seed scatter can be reduced using great care; however, for small-seeded species, prevention of gene flow is likely to be impossible without intentional bioconfinement.

Gene confinement methods

▪ Nonbiological methods: physical, spatial, mechanical & temporal methods

Conceivably, transgenic plants can be confined spatially and temporally using nonbiological methods. Physical containment includes specific cases such as production of plant-manufactured pharmaceuticals in greenhouses, in underground facilities, inside buildings or in cultivation areas unique to a specific crop (e.g., growing rice in Kansas) [27]. Biofuel feedstocks are likely to be so extensively widespread that physical containment is not feasible [25]. Mechanical control of flowering would be one strategy to contain transgenes in feedsocks (e.g., pollen and seed production could be prevented by mowing perennial grasses). However, frequent mowing would be costly and subject to human error and, thus, not feasible for bioenergy feedstocks.

▪ Bioconfinement methods

The distinction between containment (a fail-safe procedure where gene flow does not occur or is so low as to be negligible) is different from the common field practice of confinement, which attempts to limit gene flow to prescribed regulatory levels or standards. The current available strategies for bioconfinement, which could be applied to perennial transgenic biofuel crops for control of gene flow, include male sterility, plastid transformation and maternal inheritance, nuclear male sterility, seed sterility, use of various recombination strategies for selected transgene removal in tissues and total sterility methods. Other possible bioconfinement strategies that might be adequate solutions for the control of gene flow in some cases are species limited and not currently amenable to molecular manipulation, such as cleistogamy, apomixis and genomic incompatibility.

Male sterility

As previously discussed, the primary route of transgene escape will be through pollen and, thus, prevention of viable pollen production represents a potential bioconfinement strategy. Indeed, there has been much research on engineering male sterility for hybrid plant production [28], bioconfinement [29] and other purposes [30]. One target for male sterility is the tapetum, the innermost layer of the anther wall that surrounds the pollen sac, which is needed for pollen development. A variety of anther- and tapetum-specific genes have been identified that are involved in normal pollen development in many plant species, including maize [31], rice [32], tomato [33], *Brassica campestris* [34] and *Arabidopsis thaliana* [35]. Selective ablation of tapetal cells by cell-specific expression of nuclear genes encoding cytotoxic molecules [28,36–39] or an antisense gene essential for pollen development [29,35,36] blocks pollen development, giving rise to stable male sterility.

To induce male sterility in turfgrass, the 1.2-kb rice *rts* gene regulatory fragment was fused with two different genes **(Figure 1)** [40]. One was the antisense of rice *rts* gene that is predominantly expressed in tapetum cells during meiosis. Another gene was the *Bacillus amyloliquefaciens* ribonuclease gene *barnase*, which ablates tapetal cells by destruction of RNA [41]. Both of these approaches have been shown to be effective in various plant species [28–30,42]. Field performance of these plants resulted in the recovery of three herbicide-resistant plants from over 10^5 tested wild-type seeds [Kausch AP, Unpublished Data] indicating low leakage of the system. There is currently no acceptable standard for gene flow frequency in such a case. Without prescribed limits for a given crop and transgene combination for gene flow, it is difficult to understand the functional adequacy of any sterility system. Nonetheless, nuclear male sterility, resulting in the lack of viable pollen grains when linked to the genes of agronomic interest, provides an important tool to study effective mechanisms for interrupting gene flow. In addition, male sterile lines will provide important breeding tools. However, previous experience with this system has raised questions about the efficacy of this system. Although tapetal-targeted barnase expression can induce male sterility, it has been found that tapetal cell lysis can be incomplete under certain conditions, which leads to a leaky and partially male fertile phenotype. The barnase system has yet to be used as a biocontrol system for any commercial crop and might not provide sufficient prevention.

Cytoplasmic male sterility & plastid transformation technologies

Cytoplasmic male sterility (CMS) and plastid transformation also offer choices for controlling gene flow between dedicated energy crops and their wild relatives. CMS is caused by mutations in the genomes of either the plastid or the mitochondria and are exclusively maternally inherited in many plant species. In crop plants, nuclear genes that restore fertility have been widely applied for creating hybrids. Consequently, the development of CMS systems for dedicated energy crops would be useful for gene confinement as well as

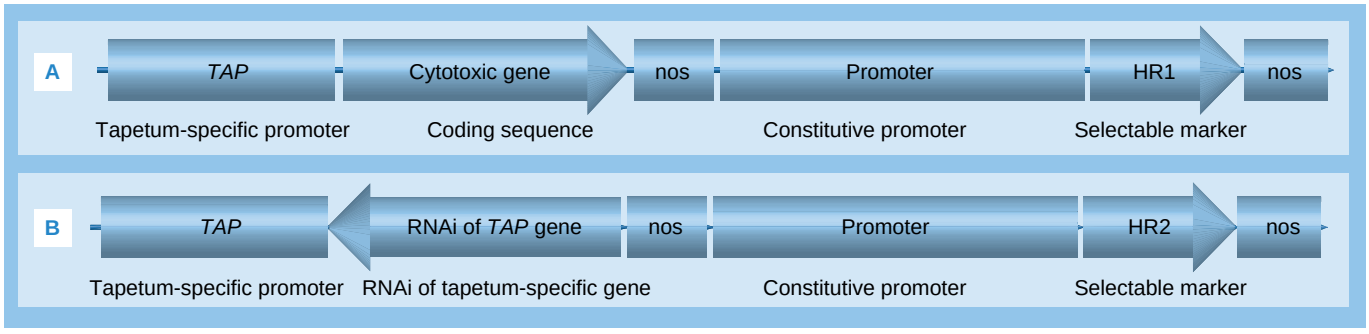

Figure 1. Nuclear male sterility is induced by tapetal ablation, using a tapetum-specific promoter, driving expression of either (A) a cytoxic gene (i.e., barnase) or (B) the antisense of the native gene with selection via herbicide resistance.

Key term

Transgene bioconfinement: Measures and traits used to prevent genetically modified organisms and their transgenes from entering the environment through nontransgenic or wild relatives

providing valuable breeding tools for these crops by allowing the development of recurrent inbred lines. However, the current status of breeding efforts for these crops does not yet include these tools. An attractive option would be to genetically engineer a CMS-associated mitochondrial gene for stable nuclear expression, such that pollen production would be disrupted [43].

The first engineered cytoplasmic male sterility system in plants was accomplished by expression of β-kethiolase by stable integration of the *phaA* gene via the plastid genome [44]. Prior attempts to express the *phaA* gene in transgenic plants were unsuccessful. The *phaA* gene was efficiently transcribed in all tissue types including leaves, flowers and anthers. Coomassie-stained gel and western blots confirmed hyperexpression of β-ketothiolase in leaves and anthers, with proportionately high levels of enzyme activity. The transgenic lines were normal, except for the male sterile phenotype, lacking pollen. Scanning electron microscopy revealed a collapsed morphology of the pollen grains. Floral developmental studies revealed that transgenic lines showed an accelerated rate of anther development, affecting their maturation and resulting in aberrant tissue patterns. Abnormal thickening of the outer wall, enlarged endothecium and vacuolation affected pollen grains and resulted in the irregular shape or collapsed phenotype. This method offers yet another tool for transgene containment and provides an expedient mechanism for F1 hybrid seed production.

Integration of transgenes into the plastid genome is an approach to accomplish both **transgene bioconfinement** and high levels of transgene expression without the possibilities for gene silencing or position effects [45,46]. The nearly complete maternal inheritance of genetically modified plastid genomes and the absence of any reproductive structures when foreign proteins expressed in leaves are harvested offer an efficient transgene confinement system via pollen or seeds and facilitates their safe production in the field [46,47]. Two recent studies point out efficient control of maternal inheritance of transgenes in transplastomic tobacco. Ruf *et al.* set up a stringent selection system for paternal transmission by using male sterile maternal parents and transplastomic pollen donors conferring plastid-specific antibiotic resistance and green fluorescence for visual screening [48]. This selection system identified six among 2.1 million seedlings screened (frequency of 2.86×10^{-6}) that showed paternal transmission of transgenes and the authors concluded that plastid transformation provides an effective tool to increase biosafety of GM crops. Therefore, transplastomic plants producing human therapeutic proteins have been already tested in the field after obtaining USDA–APHIS approval [49] but there remains uncertainty regarding the acceptable level of gene flow when applied to perennial plants that will be used on a large scale for biofuel production [50,51].

While not offering absolute transgene containment, confining transgenes within plastid genomes will greatly limit the passage of transgenes via pollen and therefore to other crops or relatives via outcrossing. However, if transgene products are harvested from leaves before the appearance of any reproductive structures, absolute transgene containment via pollen or seeds is possible. The major technical challenge to this possible containment strategy is to get the transgene into every plastid (homoplasmy) in each cell. However, only three rounds of selection on regeneration media are typically required to reach homoplasmy in tobacco [44]. Southern blots and PCR are used to measure if any wild-type copies are present and homoplasmic lines can be identified and increased. Since plastids are prokaryotic compartments, they lack the silencing machinery found within the cytoplasm of eukaryotic cells. Each plant cell contains 50–100 plastids and each plastid contains approximately 100 copies of its genome, so it is possible to introduce 20,000 copies of the transgene per cell. Transgenes have been stably integrated and expressed via the tobacco plastid genome to confer important agronomic traits, including herbicide, insect and disease resistance, drought and salt tolerance, cytoplasmic male sterility or phytoremediation [45]. Plastid genomes of several crop species, including cotton, soybean, carrot, sugarbeet, cauliflower, cabbage, oilseed rape, poplar, potato, tomato, tobacco, lettuce and other crops, have been also transformed [45]. A total of 24 vaccine antigens against 16 different diseases and 12 biopharmaceuticals, including insulin and interferon, have been expressed in tobacco plastids and many are fully functional [45,52]. Complete plastid genome sequences of more than 30 crop species have been determined recently, facilitating rapid advancement in this field [53]. Plastid transformation in cereal crops is feasible but it should be developed in dedicated energy crops (e.g., perennial grasses, sorghum and maize) [54].

Biofuel production from lignocellulosic materials is limited by the lack of technology to efficiently and economically release fermentable sugars from the complex multipolymeric raw materials. Therefore, mixtures of enzymes containing endoglucanases, exoglucanase, pectate lyases, cutinase, swollenin, xylanase, acetyl xylan esterase, β-glucosidase and lipase genes from bacteria or fungi have been expressed in tobacco plastids [55]. Homoplasmic transplastomic lines showed normal phenotype and were fertile. Plastid-derived crude-extract enzyme cocktails yielded more (up to 3625%) glucose

from filter paper, pine wood or citrus peel than commercial cocktails and were 1000–3000-fold cheaper than recombinant commercial enzymes [55]. Although individual enzymes have been expressed in plants before, this is the first report of production of recombinant enzyme cocktails from transgenic plants. Transgene containment is a serious concern in transgenic plants expressing cell wall-hydrolyzing enzymes via the nuclear genome, because of their potential for introgression to out-crossing crops or weeds, and therefore biological confinement via maternal inheritance may present one viable method for the control of gene flow.

Seed-based gene confinement

Seed-based bioconfinement relies on the use of genetic use restriction technologies (GURTs). Various forms of genetic use restriction are already widely in use in agriculture, such as those based on sterile F1 hybrids (seedless fruits), nonpropagable hybrid maize, a mainstay of US agriculture, and hybrid rice, which has increased yields in east Asia. Even though this apparently biased terminology emphasizes only the proprietary protection issues of corporate interests, perhaps the most impactful use of GURTs is related to transgene bioconfinement. There are two major classes of GURTs: varietal-level GURTs (V-GURTs) and trait-specific GURTs (T-GURTS), which correspond to growth stages that trigger a genetic switch for containment. V-GURTs allow for normal growth and full development of the desired seed; however, the progeny seed, if planted, will not germinate. Gene containment is achieved by the inability of the plants that contain the activated V-GURT mechanism to produce viable progeny, either through the pollen or via seed. T-GURT systems regulate trait expression, making the value-added trait (transgene) available only if the farmer triggers the genetic switch mechanism. Plant function is normal, but when a particular engineered trait is needed in a farmer's field, a specific triggering chemical is applied to activate transgenes expressing a desired characteristic (e.g., insect resistance). Transgene bioconfinement would be achieved by the inability of the plants to express the transgenic trait in the absence of the activating chemical that is not indigenous in the environment.

Bioconfinement & public perception of GURTs

After the issue of the original GeneSafe patent, which described a V-GURT mechanism that results in nongerminable seeds as a means of gene confinement [101–103], several controversies ensued, along with the Rural Advancement Foundation International (now the ETC Group) nomme fatal 'Terminator.' The GeneSafe technology utilizes an inducible system for the activated expression of a recombinase (i.e., *Cre*), which can be applied prior to seed germination for rendering a nonviable seed **(Figure 2)**.

One of the major issues raised in objection to the use of V-GURTs is the possible impact on seed viability in compatible nontransgenic or T-GURT crops in neighboring fields as a result of the spread of pollen from a V-GURT crop. V-GURTs are currently time designed for use in crops that preferentially self-pollinate rather than out-cross (e.g., cotton, soybean and wheat) [103]. In such cases, negative effects on neighboring fields would be very restricted and would not be detectable above the background of normal germination rates for field-grown crops. V-GURTs targeted for crops that

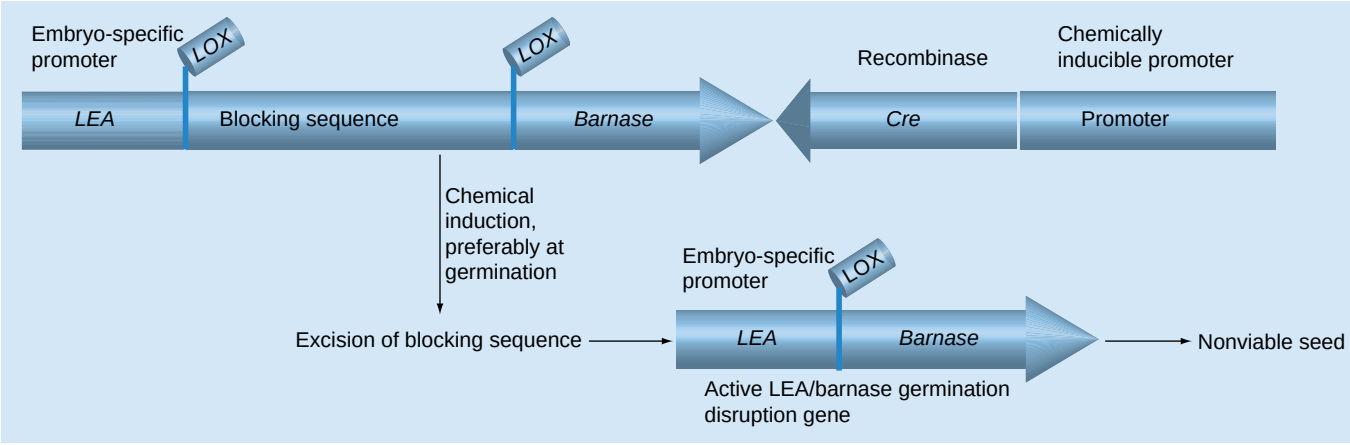

Figure 2. GeneSafe™ technology uses a seed-treatment chemical induction, resulting in nonviable seeds. An inducible promoter is activated by applied treatments prior to germination, which activates expression of *Cre*, causing excision of a 'blocking sequence' flanked by *LOX* sites and juxtaposing the embryo-specific expression (driven by a LEA) of a cytoxic gene (*Barnase*), resulting in sterile seeds [101–103].
LEA: Late embryogenic abundant promoter.

readily out-cross would have to contain design elements for the removal of transgenes during microsporogenesis, so as to prevent transgene escape via pollen dispersal. Such mechanisms have been proposed that rely on site-specific recombination systems for transgene excision [101–103] but transgene exclusion might also be accomplished using pollen-specific gene expression of a cytoxin gene, such as barnase, and transgene elimination would be accomplished in heterozygous plants. A similar concern has been posed with regards to the possibility that pollen from V-GURT plants could prevent germination of seeds in neighboring related wild species and, thus, reduce their long-term viability in the native habitat. Obviously, preventing the germination of hybrid seed developed from pollen outflow from a crop to a wild species is a premium outcome in the desire to contain transgenes in the environment, but it would be problematic if the long-term viability of a wild species is affected. In realistic terms, this is an unlikely scenario, because such an outcome would require that the wild species was completely compatible with the crop containing the V-GURT and that non-V-GURT pollen was absent from the environment (i.e., no genetic barriers between types). Most crops do not have relatives that are sexually compatible in agricultural areas and hybridization is rare. In cases where there is a measure of compatibility and a problem exists, a change in the design of the V-GURT may be warranted.

Varietal-level GURTs have also been criticized for their supposed potential for socio-economic impacts on agriculture in developing countries. The nongerminability of GeneSafe seeds and the resultant need to purchase new seed for the planting of a new crop has been suggested to be an unfair economic burden on small farmers, especially those engaged in subsistence farming. Although it is true that farmers would be required to purchase new seed every year, one has to bear in mind that GeneSafe and other V-GURT technologies alone have no value and would only be in a crop in conjunction with a valuable or advantageous transgenic trait (i.e., V-GURTs and the trait are linked). Indeed, GeneSafe technologies would allow subsistence farmers access to superior traits that would have the potential to insure and increase yields and thus deliver them from the vagaries of the environment within which they practice, perhaps to the point of enabling the establishment of a production-level operation.

Environmental concerns have been raised that the method used to prevent the germination of activated V-GURT seeds could harm other organisms. The currently used gene products disrupt seed metabolism; they are not toxic to animals and occur naturally in plants and microbes that are normally consumed in animal diets. Similarly, the chemical seed treatment used to activate the V-GURT during stand establishment would have to be, by necessity, environmentally friendly or neutral. The use of tetracycline described in the GeneSafe prototype was never targeted for commercial use in the field.

Transgenic seedless fruits (although not a complete gene-containment technology) described by Tomes *et al.* [104] and the GeneSafe technologies of Oliver *et al.* [101–103] are all V-GURTs designed to prevent gene out-flow from transgenic plants via seeds. The basic strategy outlined in these patents is to control the activation of a 'germination-disruption gene' such that its expression prevents establishment of the next generation of a crop that bears a value-added or production-benefit transgene. The gene activation is timed such that the transgene is available in an uncontained environment, such as a farmer's field, and it is only after a crop is produced that the activated germination-disruption gene is expressed and effective. The mechanism is also designed such that pollen from a plant that contains the activated germination-disruption gene fertilizes an ovule and generates a nongerminable seed. Although this is desired for total gene containment, this could be problematic in an open pollination scenario. The GeneSafe mechanisms described here were designed for crops that reproduce under restricted or mainly closed pollination. The three elements needed for GeneSafe are: a promoter that responds to a specific exogenous stimulus, a site-specific recombinase to remove a physical block, and a seed-specific promoter that is only active late in seed development. These elements were used to generate two genetic systems (basic systems from which refinements can be added): one based on a repressible promoter mechanism that is relieved by exposure to an activator and the other, a more simple system, based on a chemically inducible promoter. These two mechanisms were originally designed for use in GM cotton as a technology-protection system [101–103].

At the present time, the repressible GeneSafe technology has been developed in both cotton and tobacco to varying degrees, tobacco being the most advanced [102]. Germination tests of seed derived from selfing seedling activated (tetracycline-treated) dual hemizygous plants that exhibit precise excision in vegetative cells of the plants did not generate the expected 3:1 ratio of nongerminable to germinable seed (assuming successful activation of *CRE* in all germline cells of the parental lines). In fact, in only a few cases were germination percentages reduced. However, PCR analysis of the seeds used in the germination tests revealed that all were either heterozygous for the excision phenotype or homozygous for the intact module; no seeds homozygous for the excision event were detected (360 seed lots tested so far [Oliver M *et al.*, Unpublished Data]). The implication

is that seeds that contain two copies of the excision event do not develop to maturity in tobacco pods of plants derived from tetracycline-treated seeds. This would further imply that the timing of expression of the protein synthesis inhibitor driven by the cotton late embryogenesis abundant (LEA) promoter in tobacco does not mimic that seen in cotton (i.e., it occurs prior to the maturation phase of seed development) and that the level of expression of the protein synthesis inhibitors suffices to affect viability when only one copy of the gene is present. Research is ongoing in this pilot study.

Gene deletor system

A highly efficient system to delete all transgenes from pollen or both pollen and seed has been developed [56]. In this method, transgenic cassettes are effectively excised using components from both FLP/*FRT* and *CRE*/*loxP* recombination systems **(Figure 3)**. When *loxP-FRT* fusion sequences (86 base pairs) were used as recognition sites, simultaneous expression of both *FLP* and *CRE* reduced the average excision efficiency, but the expression of either *FLP* or *CRE* alone increased the average excision efficiency. When three different gene promoter sequences were used to control the expression of the *FLP* or *CRE* gene, transgenic tobacco events with 100% efficiency in transgene deletion from pollen or both pollen and seed were observed, based on analysis of more than 25,000 T1 progeny. The deletion of all functional transgenes from pollen or both pollen and seed was confirmed using three different techniques: histochemical β-glucuronidase (GUS) assays, Southern blot analysis and PCR. These studies were conducted in tobacco under greenhouse conditions and have not yet been field tested. The gene-deletor system, which can produce 'nontransgenic' pollen and/or seed from transgenic plants, may provide a useful bioconfinement tool for transgenic crops and perennials and may be applicable for vegetatively propagated biofuel plants. If a conditionally inducible gene promoter, such as a chemically or high temperature-inducible or postharvest-stage active promoter, were used to control recombinase expression, all functional transgenes could be deleted throughout the plant on application of the inducer or after harvesting.

Total sterility

Recently, Luo, Kausch, Chandlee and Oliver proposed a mechanism to eliminate all possibility for gene transfer in species that are primarily grown for their green biomass, in particular turf grasses **(Figure 4)** [Kausch A, Unpublished Data]. The strategy hinges on the prevention of flowering using a site-specific recombinase (in this case the *FLP*/*FRT* system from yeast) to activate a gene designed to downregulate a gene critical in the initiation of floral development. The targeted gene for downregulation is *FLORICAULA/LEAFY*, which triggers the vegetative-to-reproductive developmental transition of meristems. The mechanism operates by establishing a transgenic line homozygous for both the transgene of interest and a genetic construct containing the following linked elements: a constitutive plant promoter – an *FRT* site (recognition site for *FLP*) – a blocking sequence – an *FRT* site – RNAi or antisense construction for *FLORICAULA/LEAFY*. In the final seed production cycle, homozygous plants are crossed to plants homozygous for a constitutively expressed *FLP* gene to produce hybrid seed. When grown, the hybrid seeds will generate plants that constitutively express *FLP*, resulting in the excision of the blocking sequence contained in the initial construct. This will activate the constitutive expression of the RNAi or antisense construction for *FLORICAULA/LEAFY*. This in turn will downregulate the expression of the endogenous *FLORICAULA/LEAFY* genes, rendering the plant incapable of producing flowers. The vegetative growth habit of the hybrid retains its commercial application but is incapable of transferring transgenes to neighboring grasses or weedy relatives. This is in effect a hybrid total gene-containment system. Variations on this scheme are possible to include selection of the outcome using two herbicide-resistance genes ensuring the hybrid seed.

▪ Regulatory issues for perennial bioenergy feedstocks

Currently, the USDA–APHIS–BRS regulates the environmental release of transgenic plants on a case-by-case basis. Permits are required for all nonderegulated transgenic plants to be grown outside of containment greenhouses. The value of BRS to both biosafety and innovation in transgenic field testing is apparent, in that transgenic releases in the USA do not require costly permitting or undue paperwork. However, permits are often accompanied by additional requirements. For example, in the field testing of transgenic switchgrass, Stewart *et al.* are required to prevent flowering and set seed (i.e., by the mechanical removal of flowers prior to anthesis). BRS considers the planting of transgenic switchgrass, a plant with which they have little experience, to be a case that required the imposition of a stringent set of precautions to avoid gene flow when the first field tests were performed, even though the transgenic plant itself may contain only nonherbicide-selectable and scorable marker genes.

The process of US deregulation includes lengthy reviews and data collection spanning different environments over several years, with consideration of several factors including biology, geography and ecology of the

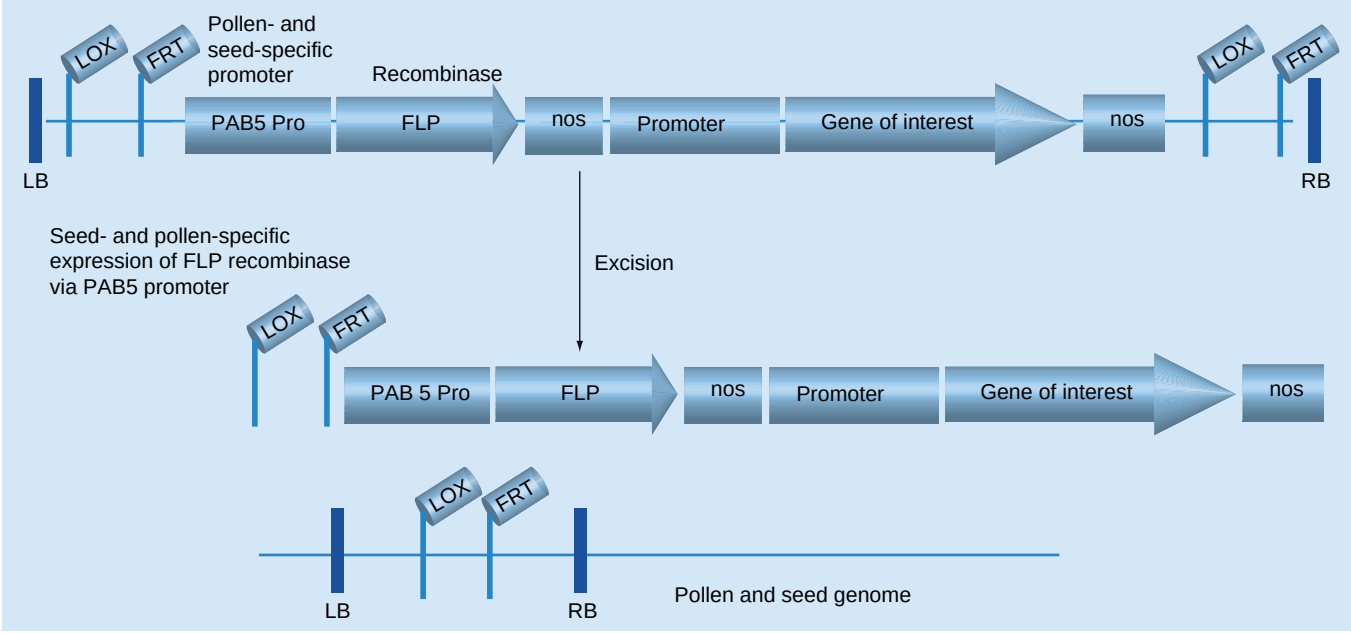

Figure 3. Gene-deletor technology improves the efficiency of excision by using *FRT* and *LOX* sites to resolve a product without inserted transgenes.
Pro: Promoter.

plant, the genes and traits of interest, the possibility of gene flow to wild and nontransgenic relatives, the possibility of weediness or invasiveness and unintended consequences to other organisms. The current US regulatory system is costly, cumbersome and lengthy owing, in part, to the split between three agencies (USDA, EPA and FDA). The high cost of deregulation deters innovative startup ventures or even medium-sized companies from entering the market and, therefore,0 tends to reinforce the dominance of the larger agrocultural

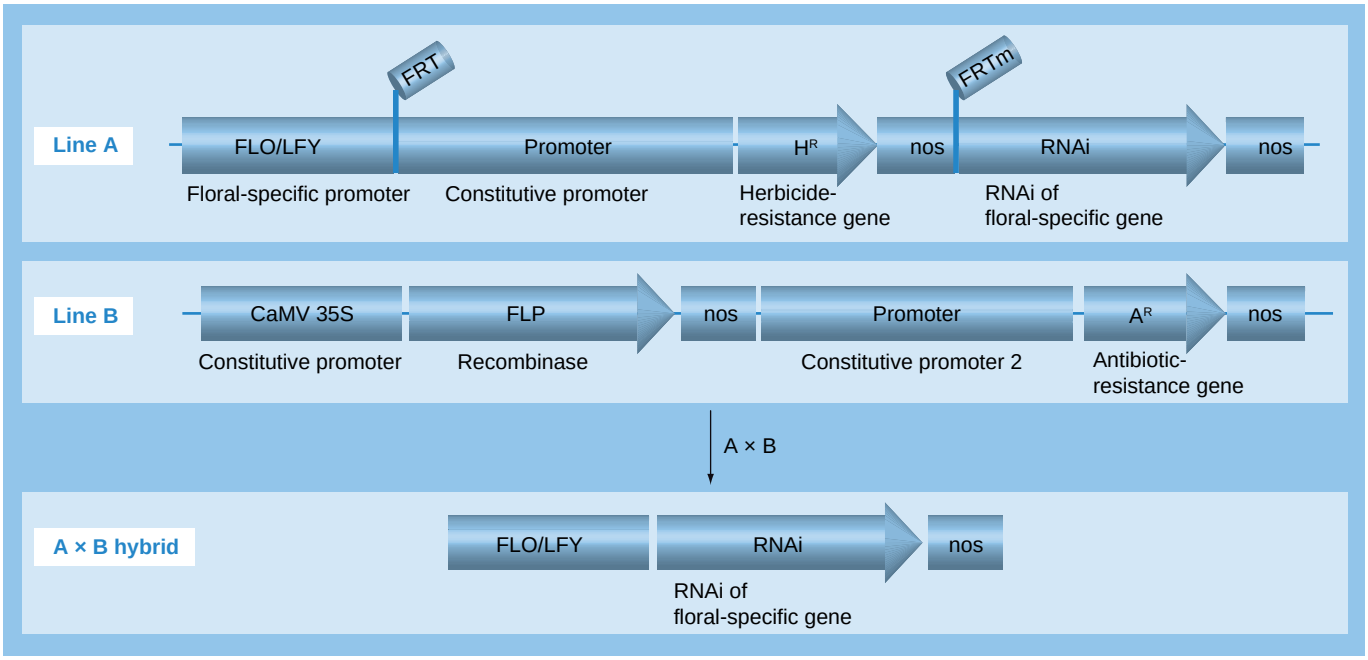

Figure 4. Strategy for conferring F_1 total vegetative growth. The A × B cross results in excision of the internal fragment flanked by *FRT* sites and juxtaposition of the FLO/LFY promoter with the FLO/LFY RNAi, causing inflorescence ablation during transition to flowering.

biotechnology companies, many of whom have relatively little interest in biofuel development. Perhaps this situation could be ameliorated by the provision of freedom to operate intellectual property packages that would streamline deregulation.

It is important to assess individual bioeneregy feedstock species independently and to evaluate the introduced traits or characteristics to determine if they could enhance the vigor or invasiveness of wild or weedy relatives or have other detrimental effects. While some traits may pose relatively few risks (e.g., herbicide tolerance), others might have the potential for unintended consequences and invasiveness (e.g., drought and pest tolerance). Most of the next-generation dedicated energy crops will be perennial trees and grasses [8]. Many species that are being seriously considered to play a major role in the developing biofuels industry have wild relatives in the regions where they will be grown. In addition, for some prominent feedstocks, such as switchgrass, there is an absence of data on gene flow. The regulatory data requirements or constraints for gene flow are still unclear. While one may assume that transgene containment is the goal, acceptable levels of transgene escape need to be practically defined and the concerns about gene flow need to be balanced against the studied consequences of gene flow in field trial studies. Considering the cost of deregulation and the subsequently imposed market restrictions, the risks and benefits of some regulatory requirements may need to be reconsidered (i.e., modified without unduly compromising safety); for example, deregulation of the transgenic process itself, creation of regulatory classes in proportion to potential risk, exemption of selected transgenes and classes of transgenic modifications and elimination of the event-specific basis of transgenic regulation [57]. Even though the USA has led the way in the innovation and implementation of GM technologies to date, we expect the usefulness of this technology to spread to applications in dedicated biofuels crops that will be grown and used internationally. Therefore, international negotiations to harmonize and stimulate trade, via the World Trade Organization, for example, are probably almost as important for the future health of the biofuels sector as are technological advances. Most probably, without a robust strategy for bioconfinement transgenic traits introduced into these crops will never be released for practical applications.

Future perspective

Biotechnology will undoubtedly play a large role in the development of a successful cellulosic energy industry. Transgenic feedstocks will likely be required to boost biomass yields above an economic threshold and, most importantly, to deal with the recalcitrance problem of cell wall hydrolysis [8]. For example, in a model plant, there is a clear negative relationship between lignin and saccharification [58]. Lignin occludes hydrolytic enzymatic access to cellulose and hemicellulose. Therefore, decreasing lignin biosynthesis in feedstocks will likely play an important role in decreasing costs for cellulosic biofuel production. There are many reasons to believe that decreasing lignin will also decrease plant fitness. Lignin plays important roles in plant cell wall integrity and strength and also in plant defense. Thus, some transgenic traits (increased biomass) might provide a selective advantage and persist in nature. Others (decreased lignin) will be selected against and likely disappear over time. A recent proposal was made to essentially leapfrog cellulosic biofuels, especially ethanol, to use dedicated bioenergy to convert into electricity for next-generation plug-in electric vehicles [59]. The argument is one of higher efficiency through fewer energy losses in the conversion of biomass to usable energy for transportation. If this is the subsequent dominant path for dedicated bioenergy feedstocks, then higher yield and stress tolerance will likely be of more importance than cell wall traits.

Biofuel production from lignocellulosic materials is limited by the lack of technology to efficiently and economically release fermentable sugars from the complex multipolymeric raw materials. Because of variation in cell wall composition in different biomass (e.g., wood, citrus peel and corn stover), there is a great need to produce individual enzymes and enzyme cocktails in an economic manner. Enzymatic hydrolysis of biomass reduces or eliminates expensive pretreatment requiring high energy or releasing toxic chemicals or reagents as byproducts. Unfortunately, enzymes currently produced in the fermentation system are highly expensive and are not competitive with fossil fuels. Therefore, there is a great need to produce biomass-hydrolyzing enzymes in plants [9]. Transgene containment will be a serious concern in transgenic plants expressing cell wall-hydrolyzing enzymes via the nuclear genome because of their potential toxicity to out-cross crops or weeds and, therefore, biological containment via maternal inheritance or other strategies or product harvest before appearance of any reproductive structures are essential for effective bioconfinement.

There are numerous research programs working on improving biotechnology and genomic resources of feedstocks, but the requirements for deregulation and commercialization of these crops remain uncertain. Regardless of the transgene, we believe that bioconfinement will likely be a desirable strategy to limit trangene flow from commercial transgenic (and nontransgenic) feedstock candidates. Depending on the biology, ecology, geographic distribution and flowering phenology

of the candidate biofuel species and their compatible relatives, it is important for developers and regulators to study and determine levels of gene flow tolerance and thereby minimize potential adverse ecological consequences of gene flow.

We have discussed the available strategies for transgene bioconfinement that are currently under development. There are limitations to most of these strategies, notably, physical, spatial, mechanical and temporal containment. In addition, some of the sophisticated biotechnology methods are not perfected or adapted for bioenergy feedstocks. Biotechnology specifically for bioconfinement is in the early stages of development and there are many choices with regard to components. Pollen sterility has been accomplished in a number of species but there are not many systems that have proven to be effective and all are patented and therefore might not be available. Certainly, additional male sterility systems are needed. Male sterility should be sufficient for mitigating gene flow in many cases, as wild-type crosses would produce progeny that would also be male sterile, but any system should be rigorously tested in the field for the species of interest. Very little is known about the frequency of reversion of these mechanisms (i.e., ribonucleases) to fertile phenotypes. CMS systems would provide a similar level of bioconfinement but, again, additional technologies are needed to enable the necessary freedom-to-operate that would spur development.

Maternal inheritance through plastid transformation has been developed for several dicot plants including cotton, soybean, carrot, cauliflower, sugarbeet, cabbage, oilseed rape, poplar, potato, tomato, tobacco, lettuce and other crops. Among various bioconfinement strategies developed so far, plastid transformation has been developed in more crop species than any other system. Plastid genomes have been engineered to confer the highest levels of expression for several agronomic traits, including herbicide, insect and disease resistance, drought and salt tolerance, cytoplasmic male sterility or phytoremediation [45]. Plastid transformation in cereal crops is feasible but it should be developed in dedicated energy crops (e.g., perennial grasses, sorghum and maize) [54]. When transgene products are harvested from leaves before the appearance of any reproductive structures, absolute transgene containment via pollen or seeds is possible. In current field trials, USDA–APHIS must be notified prior to harvest and field inspectors must investigate the appearance of reproductive structures; therefore, regulations are already in place to evaluate transgene containment for this technology and several products have been tested in the field [49].

The GeneSafe technology and other seed-based GURTs offer conditional lethality, which can be chemically induced to prevent flowering or seed development. These technologies require complete biological induction and have human management drawbacks. However, these methods provide solutions that will allow production of seeds that will contain the trait of interest and prevent the escape of nonfunctioning transgenes. Currently, these approaches are considered to be the best and only strategies that could be deployed to prevent seed-based gene flow. The possibility of creating a two-component bioconfinement component system whereby crossed progeny produce seed that will never germinate and result in total sterility might offer promise for transgenic perennial feedstocks. It also might be desirable to include failsafe and backup mechanisms to decrease gene flow even further than that accomplished using single systems.

The potential benefits from biotechnology for the next-generation of crops are many [3]. We must remember the lessons we have gleaned from food and fiber crop and from the transgenic creeping bentgrass experiments, especially from the regulatory perspectives. Moving forward, landscape-level field testing and monitoring of genetic containment systems for perennial bioenergy feedstock crops must be accomplished to determine their efficacy. This should include a comprehensive and workable set of guidelines established by regulatory agencies, which outlines acceptable levels of gene flow.

Dedication

This paper is dedicated to the memory of Peter Mascia (1950–2009) who contributed significantly to this manuscript prior to his passing on 28 May 2009.

Executive summary

- Transgenic traits will likely be an important tool to improve biofuels crops.
- In order to achieve the full benefit of transgenic tools applied to perennial biofuels crops, a robust bioconfinement strategy will need to be in place to mitigate gene flow to wild and nontransgenic relatives.
- Strategies for gene confinement of transgenic perennial biofuels crops include physical, spatial and temporal confinement, maternal inheritance, nuclear male sterility, seed sterility, use of various recombination strategies and total sterility methods.
- All of the currently available strategies for bioconfinement have inherent drawbacks and may require failsafe backup strategies in combination.
- A level of tolerance for gene flow from these crops requires further discussion.

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▪ Patents

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