Chapter 11
Genetic Modification in Dedicated Bioenergy Crops and Strategies for Gene Confinement

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11.1 Introduction

The utilization of dedicated crops as a source of bioenergy from renewable biomass resources is a goal with great relevance to current ecological, economic, and national security issues on a global scale. In the US, the Energy Policy Act of 2005 (EPAct 2005) issued a mandate for the use of up to 7.5 billion gallons of renewable fuel in gasoline by 2012. These amounts will likely increase in the future as a shift occurs toward renewable energy sources and away from foreign oil supplies (Robertson et al. 2008). Current strategies for liquid fuel production utilize fermentation of plant-derived starches and sugars to ethanol, mostly from grain and other food crops. One concern is whether sufficient amounts of these feedstock materials can be supplied without impacting the cost of agricultural land, competing with food production, and harming the environment. For a variety of reasons, production of fuel from dedicated non-food crops as cellulosic sources, such as

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switchgrass, Energy Cane, sorghum, Miscanthus, willow, and poplar, is widely understood as a necessary development (Sticklen 2008).

The genetic improvement of food crop species using biotechnology is well-established and, together with conventional breeding efforts, can be used to confer valuable traits. Trait enhancement and new varietial development will be useful toward the improvement of dedicated bioenergy crops. In addition, biofuels-specific traits, such as production of cellulases and other hydrolytic enzymes and biopolymers, increased cellulose, and decreased lignin can be engineered to increase fuel production per acre (Sticklen 2008). Efforts toward genetic engineering of cellulosic feedstock crops used for bioenergy have barely begun and offer significant potential improvements; however, these modifications present significant public and regulatory concerns. Commercial-scale production of some transgenic plants could lead to undesirable environmental and agricultural consequences (Altieri 2000; Dale 1993; Robertson et al. 2008; Snow and Moran Palma 1997) including transgene escape to wild and non-transgenic relatives. Thus, to realize the full potential of agricultural biotechnology for dedicated energy crops enhancement, the ecological, economic, as well as commercial impacts of gene flow must be addressed.

Currently, strategies using plant genetic engineering for biofuel production are being developed with the goal of renewable and affordable cellulosic ethanol production. Most of the plants considered as top choices for cellulosic biomass are perennial and/or have wild relatives in the areas where they will be produced commercially. Bioconfinement of engineered genes and plants used for cellulosic biofuels will likely be a prerequisite for deregulation and commercial production of these plants (Stewart 2007). Current information strongly indicates the potential for gene flow in open pollinated genetically modified (GM) bentgrass (Belanger et al. 2003; Mallory-Smith and Zapiola 2008; Reichman et al. 2006; Watrud et al. 2004; Wipff and Fricker 2001; Zapiola et al. 2007, 2008) and the need for robust gene confinement strategies (Dunwell and Ford 2005). In this chapter, we review currently viable strategies for the control of transgene flow in perennial grasses that may be useful in the engineering and commercial release of perennial dedicated biofuels crops.

11.2 Methods for Gene Confinement in Genetically Engineered Plants

11.2.1 Physical, Spatial, Mechanical and Temporal Control

One convenient method that has been proposed for gene confinement of genetically modified perennial plants would utilize agronomic practices, including physical, spatial, mechanical or temporal control. Physical containment has been proposed for specific containment requirements, such as production of plant-based

biopharmaceuticals in greenhouses, underground facilities, and growth rooms, and is suitable for some crops (tomatoes, lettuce) and for research purposes, but has serious large-scale limitations for most biofuels crops (Dunwell and Ford 2005). Spatial, mechanical or temporal control strategies have been considered for genetically modified perennial plants that could be grown in areas that are outside their normal range, or in areas where there are no wild relatives. In many ways this is similar to the current large-scale control of gene flow in maize. Genetically modified perennial grasses could be routinely mowed such that they never produce fertile flowers. In addition, GM grasses could be grown in areas where their flowering time does not match that of local species. All of these mechanisms rely on human management and thus eventually will be flawed. The consequences of gene flow that have relied on management practices have already been observed in the release of open-pollinated GM creeping bentgrass in Oregon (Reichman et al. 2006; Watrud et al. 2004; Zapiola et al. 2007, 2008).

11.2.2 Pollen Sterility

Pollen-mediated transfer is widely believed to be the major contributor to gene flow in flowering plants. Interfering with the development of male reproductive structures through genetic engineering (GE) has been widely used as an effective strategy for production of male sterility in plants. These methods are distinctly different from cytoplasmic male sterility (CMS) and shown to be extremely effective and stable. The tapetum is the innermost layer of the anther wall that surrounds the pollen sac and is essential for the successful development of pollen. It has been shown that the tapetum produces a number of highly expressed messenger RNAs. Genes expressed exclusively in the anther are most likely to include those that control male fertility. Indeed, a variety of anther- and tapetum-specific genes have been identified that are involved in normal pollen development in many plant species, including maize (Hanson et al. 1989), rice (Zou et al. 1994), tomato (Twell et al. 1989), *Brassica campestris* (Theerakulpisut et al. 1991), and *Arabidopsis* (Xu et al. 1995). Selective ablation of tapetal cells by cell-specific expression of nuclear genes encoding cytotoxic molecules (Goetz et al. 2001; Jagannath et al. 2001; Mariani et al. 1990; Moffatt and Somerville 1988; Tsuchiya et al. 1995) or an antisense gene essential for pollen development (Goetz et al. 2001; Luo et al. 2000; Xu et al. 1995) blocks pollen development, giving rise to stable male sterility.

To induce male sterility in turfgrass, the 1.2-kb rice *rts* gene regulatory fragment, TAP (Lee et al. 1996) was fused with two different genes. One was the antisense of the rice *rts* gene that is expressed predominantly in the anther's tapetum during meiosis. Another was a natural ribonuclease gene from *Bacillus amyloliquefaciens* called *barnase*, which ablates cells by destruction of RNA (Hartley 1988). Both of these approaches have been shown to be effective in other plant species (De Block et al. 1997; Higginson et al. 2003; Luo et al. 2000; Mariani et al. 1990; Yui et al. 2003). Separately, both chimeric gene constructs were linked in a tandem construct

to the *bar* gene driven by either a rice *ubi* promoter or the CaMV35S promoter for selection by resistance to the herbicide phosphinothricin. These two constructs—pTAP:*barnase*-Ubi:*bar* and pTAP:a*rts*-35S:*bar*—were introduced separately into bentgrass (*Agrostis stolonifera* L.), *cv* Penn-A-4 using *Agrobacterium tumefaciens*-mediated transformation. Transgenic plants were screened from a population of independent transformation events recovered by phosphinothricin (PPT) selection. A total of 319 primary transgenic callus lines (123 from pTAP:*barnase*-Ubi:*bar* transformation and 196 from pTAP:a*rts*-35S:*bar* transformation) were recovered and regenerated into plants. Under greenhouse conditions, the insertion and expression of the two gene constructs did not affect the vegetative phenotype. The transgenic plants were vigorous and morphologically indistinguishable from untransformed control plants. PCR assays and Southern blot analysis on genomic DNA from independent transgenic plants were carried out to assess the stability of integration of the transgenes in the host genomes. The *bar* gene was present in all the transformants, and the *barnase* or antisense *rts* gene was also detected in the respective transgenic plants. All the transgenic events had less than three copies of the inserted transgene, and a majority of them (60–65%) contained only a single copy of foreign gene integration with no apparent rearrangements.

To check the sterility/fertility status of pollen from various transgenic plants expressing *barnase* or antisense *rts*, vernalized transgenic and non-transgenic control plants were grown in the greenhouse and flowered at 25°C in artificial light under a 16/8 h (day/light) photoperiod. The pollen was taken 1 day before anthesis for viability analysis using iodine-potassium iodide (IKI) staining. More than 90% of the plants (20/23) containing *barnase* and around 50% of the plants (40/79) containing the antisense *rts* gene were completely male-sterile, without viable pollen, which are normally stained darkly by IKI as observed in the wild-type control plants and *hygromycin*-resistant control transgenic plants that do not contain the *barnase* or the antisense *rts* gene, indicating that cell-specific expression of the *barnase* or the antisense *rts* gene in transgenic plants blocks pollen development, giving rise to male sterility. Light microscopy of cross-sections through flowers at anthesis showed that tapetum development had been interrupted resulting in aborted pollen maturation. Interestingly, the single gene knockout phenotype achieved through the antisense approach appears developmentally different from barnase ablation, but both have resulted in 100% stable male sterility. Therefore, when linked to genes of agronomic interest, nuclear male sterility resulting in the lack of viable pollen grains provides an important tool to study effective mechanisms for interrupting gene flow.

11.2.3 Cytoplasmic Male Sterility, Chloroplast Transformation and Maternal Inheritance

A major concern in GE perennial grass development is the possibility of the GE trait escaping into other crops or wild/weedy relatives. The use of inherent systems, such

as Cytoplasmic Male Sterilty (CMS), and/or GM approaches, such as Chloroplast transformation, may offer attractive solutions for controlling gene flow between dedicated energy crops and their wild relatives. CMS is caused by mutations in the genomes of either the chloroplast or the mitochondria and is thus inherited only maternally in many plant species. In many crop plants, nuclear genes that restore fertility (Rf) have been applied for creating hybrids. Consequently, the development of CMS systems for dedicated energy crops would be useful for gene confinement as well as providing valuable breeding tools for these crops. However, the current status for breeding 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 that would cause pollen disruption (He et al. 1996).

Another GM method of gene confinement that attempts to address this concern involves introducing the transgene into chloroplasts, which are maternally inherited in most crops (Daniell 2002). In plants exhibiting *Lycopersicon*-type maternal inheritance, chloroplasts are shunted to the vegetative cell during the first pollen mitotic division in pollen formation; none are found in the generative cell from which the sperm cells arise. The paternal chloroplasts shunted to the vegetative cell are generally destroyed when the pollen tube (derived from the vegetative cell) penetrates the synergid cell prior to fertilization. Direct GE of the chloroplast genome is an advantageous approach to gene confinement since it would provide the ability for multi-gene constructs with high levels of expression without the possibility of gene silencing or position effects (Daniell 2002). However, paternal inheritance of chloroplasts has been observed in tobacco, albeit at a very low rate (Ruf et al. 2007). Additionally, there exists the possibility of transgene flow from the chloroplast to the nucleus (Stegemann et al. 2003), although it can be reasonably argued that transgenes designed to function in chloroplasts will not function if transferred to the nucleus. Thus, while not offering absolute transgene containment, confining transgenes within chloroplasts will greatly limit the passage of transgenes via pollen and therefore to other crops or relatives during outcrossing.

Chloroplast transformation not only promotes gene confinement but also confers unique molecular and expression characteristics not found in nuclear transformation. Transgenes are incorporated in a site-specific manner into "spacer DNA" within the chloroplast genome by homologous recombination using particle bombardment, thereby not disrupting native genes. The major challenge is to get the transgene into every chloroplast (homoplasmy) in each cell. However, only three rounds of selection on regeneration media are typically required to reach homoplasmy in tobacco. Southern blots and PCR are used to measure if any wildtype copies are present, and homoplasmic lines can be identified and increased. Since chloroplasts are prokaryotic compartments, they lack the silencing machinery found within the cytoplasm of eukaryotic cells. Each plant cell contains 50–100 chloroplasts and each chloroplast contains ~100 copies of its genome, so it is possible to introduce 20,000 copies of the transgene per cell as spacer DNA is present in duplicate within the chloroplast genome. This allows for very high gene expression with no silencing. For example, in overexpression of the Bt *cry 2Aa2* operon via chloroplast transformation of tobacco, nearly one half of the protein

(47%) found in leaves was foreign protein with no silencing or health effects on the plant (De Cosa et al. 2001). Other additional advantages include no position or pleiotropic effects. Thus, chloroplast transformation imparts significant advantages over nuclear transformation in addition to gene confinement. However, to date, most crops, and especially dedicated energy crops (perennial grasses, sugar cane, sorghum, maize, etc.) cannot be plastid transformed.

11.2.4 Seed-Based Gene Confinement

Seed-based gene confinement generally involves the use of genetic switch mechanisms in what have become known as genetic use restriction technologies (GURTs). This nomenclature unfortunately emphasizes only the financial or patent enforcement interests of those companies that are involved in the development of GURTs and does not reflect any of the positive aspects of their development; in particular their utility in transgene confinement. There are two major classes of GURTs, V-GURTs (varietal-level GURTs) and T-GURTS (trait-specific GURTs), which relate to the event that is triggered by the genetic switch portion of the individual technologies. When triggered, V-GURT systems prevent the propagation of the crop and its associated genetic technology without the purchase of new seed. 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 purchased from the technology provider is applied to activate transgenes expressing a desired characteristic (e.g., insect resistance). The technology would presumably be paid for and activated only when needed. Gene containment is achieved by the inability of the plants to express the transgenic trait in the absence of the activating chemical, which is presumably not freely available in the environment.

11.2.5 Perceived Risks Associated with GURTs

Since the issue of the original GeneSafe patent describing an obvious V-GURT mechanism involving the production of non-germinable seeds as a means of gene confinement, many controversies have emerged, often fueled by the ascribing of such emotion-packed monikers as "Terminator" by those opposed to the use of such mechanisms. However, almost all of these concerns present issues that are either manageable or impart a negligible risk to society, the environment, or the customer.

One of the major issues raised in objection to the use of V-GURTs is the possible impact on seed viability in compatible non-transgenic or T-GURT crops in neighboring fields as a result of the spread of pollen from a V-GURT crop. V-GURTS are at the present time designed for use in crops that preferentially self rather than outcross, e.g., cotton, soybean and wheat. In such cases, the 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 readily outcross would have to contain design elements for the removal of transgenes during microsporogenesis so as to prevent transgene escape via pollen dispersal. A similar concern has been posed in regards to the possibility that pollen from V-GURT plants may prevent germination of seeds in neighboring 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 desired outcome in the desire to contain transgenes in the environment, but it would be problematic if, in doing so, the long-term viability of a wild species could be affected. In realistic terms, this is a highly 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. Most crops do not have relatives that are sexually compatible in agricultural areas, and hybridization is very rare. In cases where there is a measure of compatibility and a problem could arise, then a change in the design of the V-GURT may be warranted (see below).

V-GURTs have also been criticized for their supposed potential for socio-economic impacts on agriculture in developing countries. The non-germinability 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, in themselves, GeneSafe and other V-GURT technologies have no value and would be in a crop only in conjunction with a valuable or advantageous transgenic trait. The farmer would not be limited to a V-GURT variety but would gain the economic value of the transgenic trait should he or she so choose. In doing so, the farmer would presumably turn a subsistence level operation into a profitable and perhaps productive concern. The initial outlay for the transgenic variety maybe a barrier to acceptance but the remedy for this problem is based on a commercial or political tenet. Another concern is that large multinational companies could monopolize seed supplies by the use of V-GURT technologies. V-GURT technologies have value only in conjunction with transgenic technologies and, as non-transgenic seed will still be freely available through public concerns, it is difficult to see how seed supplies could be monopolized. Nevertheless, GeneSafe technologies are V-GURTs that are owned jointly by the United States Department of Agriculture (USDA) Agricultural Service and a private company (Delta and Pine Land Company; http://www.deltaandpine.com), and it is the involvement of the USDA that prevents the monopolization of the technology. GeneSafe and other V-GURTs do not, in themselves, provide a competitive economic advantage. On an environmental level,

concerns have been raised that the method used to prevent the germination of activated V-GURT seeds may harm other organisms. As of yet only gene products that are not toxic to animals and occur naturally in plants and microbes that are normally consumed in animal diets have been used to disrupt seed metabolism. 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. (1998), and the GeneSafe technologies of Oliver et al. (1998, 1999a, b), are all V-GURTS and all are designed to prevent gene out-flow from GE plants. GeneSafe technology, formally the Technology Protection System (TPS), was the first gene containment V-GURT to be patented and provides a complete one-generation strategy for gene containment.

In a series of three patents, Oliver et al. (1998, 1999a, b) described two primary GeneSafe mechanisms, utilizing a single strategy, to prevent gene flow from crops where seeds are the primary production target, whether it be for food, fiber, oil, or a value-added product. The basic strategy outlined in these patents is to control the activation of a germination disruption gene sequence such that its expression prevents the establishment of a second 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 only after a crop is produced is the activated germination disruption gene expressed and effective. The mechanism is also designed such that pollen emanating from a plant that contains the activated germination disruption gene carries it to the ovule that it fertilizes to generate a non-germinable seed. Although this is desired for total gene containment, as mentioned below, this could be problematic in an open pollination scenario and so the GeneSafe mechanisms described here were designed for crops that reproduce under restricted or mainly closed pollination.

The genetic mechanisms designed to accomplish these goals utilize three basic elements: (1) a promoter that responds to a specific exogenous stimulus; (2) a site-specific recombinase to remove a physical block; and (3) a seed-specific promoter that is active only 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, simpler, system based on a chemically inducible promoter. These two mechanisms were designed originally for use in GM cotton as a technology protection system.

The original mechanism was designed as the prototypical system, and because at the time of its development there were few available chemically inducible promoters, is the one that has received most attention. The mechanism consists of two constructs or modules. The first, the LEA module, consists of a late embryogenesis abundant (LEA) protein gene promoter separated from a coding sequence for a protein synthesis inhibitor protein, either Saporin or Barnase, by a "blocking sequence", which in this case contains the gene that produces the *tet* repressor

protein, flanked by LOX sites. LOX sites are recognition sequences specific for the site-specific recombinase CRE from bacteriophage PI, which is the subject of the second construct, the CRE module (Bayley et al. 1992; Boffey and Veevers 1977; Dale and Ow, 1990, 1991). The CRE gene is controlled by a 35S cauliflower mosaic virus (CaMV) promoter modified to contain three *tet* operator sites that direct binding of the *tet* repressor protein. Binding of the *tet* repressor protein to these operator sites results in the inactivation of the CRE gene.

The requirement for the precise timing of the activation of the protein synthesis inhibitor gene (germination disruptor) after seed formation and maturation necessitates the use of a LEA promoter, in particular one taken from the family of LEA genes that expresses very late in embryogenesis. In all probability such precise timing will dictate that GeneSafe technologies will be species specific. Although it is possible that LEA promoters retain their precise timing of expression when placed in a heterologous genetic environment, it is more likely that they would not and so for practical reasons one would prefer to design a GeneSafe strategy with a time-specific LEA promoter from the target crop. The original GeneSafe technologies were designed for cotton, although an attempt was made to assemble a working prototype in tobacco using cotton LEA promoters.

To establish the full repressible GeneSafe system, plants homozygous for each module have to be crossed to form a dual hemizygous plant that contains both modules. The cross has to be performed with the CRE plant as the pollen donor in order to ensure that introduced CRE gene is exposed to the *tet* repressor protein and inactivated in the fertilized egg cell. In the dual hemizygous plant, the complete GeneSafe system is inactive; the LEA promoter cannot drive the expression of the protein synthesis inhibitor during the last stages of seed maturation because of the physical presence of the blocking sequence, and the CRE gene cannot be expressed to generate the site-specific recombinase because of the binding of the *tet* repressor protein to the embedded operator sites in the 35S promoter. This allows these plants to be propagated in order to make both modules homozygous so that commercial seed stocks can be established. Transgenes can be added to either the plants that are homozygous for both modules, or they can be linked to the LEA module during the initial transformation to ensure they segregate with the germination disruption phenotype.

To activate the GeneSafe system, tetracycline, the chemical activator, is added to imbibing seeds. The tetracycline has to be able to penetrate to the cells in the L2 layer of the developing shoot apical meristem in order to activate the germline progenitor cells. The tetracycline releases the binding of the *tet* repressor protein, thus enabling transcription from the modified 35S promoter to produce the site-specific recombinase CRE. The resultant CRE enzyme locates its specific recognition sites, LOX sites (left and right), and physically removes the DNA between them. The LOX sites have been modified such that once excision occurs it is irreversible (Albert et al. 1995). This removal of the blocking sequence containing the *tet* repressor protein gene results in the permanent formation of the developmentally programmed germination-disruption (protein synthesis inhibitor) gene driven by the LEA promoter. The germination-disruption gene encodes an enzyme

that, when expressed in the cytoplasm of a cell, prevents protein synthesis and thus growth. The enzymes targeted for use in the prototype of the GeneSafe system were saporin, an enzyme that cleaves a specific sequence in ribosomal RNA, which in turn inactivates the ribosome, and a translation attenuated (an added AUG codon upstream of the native start codon) barnase, a ribonuclease derived from the bacterium *Bacillus amyloliquefaciens* that digests all cellular RNAs, thus preventing protein synthesis. The germination-disruption gene is not active following exposure to tetracycline as it is under the control of the LEA promoter. As the germination-disruption proteins are synthesized only after storage proteins and oils are fully deposited, the quality of the seeds produced by the plant is unaffected even though their ability to germinate has been compromised. Since this system is activated in all germ line cells of the plant the pollen will also carry the constructed germination-disruption gene. Flowers fertilized by the pollen from an activated plant will therefore produce seed that also cannot germinate. This, in effect, makes an activated GeneSafe plant an evolutionary dead end (both seed and pollen are effectively non-viable) and incapable of spreading transgenes into the environment.

At the present time, the repressible GeneSafe technology is in place in both cotton and tobacco to varying degrees, tobacco being the most advanced. Dual hemizygous tobacco plants, containing both the LEA and CRE modules, have been utilized in tetracycline activation tests and are presently within a selfing scheme designed to generate plants homozygous for both modules. In cotton, homozygous parental lines for each module have been generated (Oliver et al. 1999a, b). Analysis of tobacco plants that arise from tetracycline-treated dual hemizygous seeds confirm that CRE activation has occurred, both by PCR analysis demonstrating the precise removal of the blocking sequence, and by northern analysis revealing a loss of *tet* repressor transcripts. Germination tests of the seed derived from selfing of these activated dual hemizygous plants did not generate the expected 3:1 ratio of non-germinable 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 have been detected (360 seeds tested so far). The implication is that seeds that contain two copies of the excision event do not develop to maturity in the tobacco pods of the plants derived from *tet*-treated seeds. This would further imply that the timing of expression of the protein synthesis inhibitor driven by the cotton 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 is insufficient to affect viability when only one copy of the gene is present. The analysis of these phenomena is ongoing.

The repressible GeneSafe mechanism presents some challenges within a seed production setting, the most difficult being the need to make both the LEA and CRE modules homozygous prior to transgene insertion. This can be mitigated somewhat by linking the desired transgene to the LEA module in the initial construct, but this lengthens the process to reach the desired seed production level. The solution to

these difficulties became evident with the isolation and characterization of tightly controllable chemically inducible plant active promoters (Zuo and Chua 2000; Zuo et al. 2000). By replacing the *tet* repressor system elements with a chemically inducible promoter to drive the expression of the CRE gene, the GeneSafe technology can be simplified and reduced to a single construct. As a single construct it is simple to generate homozygous plants for seed production, and the more recent chemically inducible promoters are more efficient and offer tighter control than the *tet* repressor system. The inducible GeneSafe technology is being assembled in cotton at this time.

11.2.6 Gene Deletor System

The development of a highly efficient deletion mechanism that relies on site-specific recombination for removal of transgenes has been explored. Luo et al. (2007) developed a method for directing removal of transgenic cassettes from pollen and/or seed in tobacco by designing several gene cassettes using components from both FLP/*FRT* and CRE/*loxP* recombination systems. When *loxP-FRT* fusion sequences (86 bp) were used as recognition sites, simultaneous expression of both FLP and CRE reduced the average excision efficiency, but Luo et al. (2007) report that expression of either *FLP* or *Cre* alone increased the average excision efficiency, with many transgenic events being 100% efficient based on analysis of more than 25,000 T1 progeny examined per event. The deletion of all functional transgenes from pollen and seed was confirmed using three different techniques: histochemical assay for β-glucuronidase (GUS) activity, Southern blot hybridization and PCR. These studies were conducted under greenhouse conditions and have not yet been field tested. A similar system may be used to produce 'non-transgenic' pollen and/or seed from transgenic plants and to provide a bioconfinement tool for transgenic crops and perennials, with special applicability towards vegetatively propagated plants. Pollen- and seed-specific promoters could be used to control recombinase expression, whereby all functional GM genes would be deleted from these organs. If a conditionally inducible gene promoter, such as a chemically inducible or high-temperature inducible elements or conditions such as the use of inteins, were used to control recombinase expression, all functional GM genes would be deleted throughout the plant on application of the inducer.

11.2.7 Total Sterility

The introduction of novel genes by conventional or by genetic engineering is not restricted to those plants that provide food and fiber. Because of the economic and environmental importance of forage species and turf grasses, these species have been targeted for genetic improvement by GE. Improvements such as herbicide resistance, drought resistance, disease resistance, and pest resistance have all been

suggested as targets for transgenic strategies. The difficulty with such species, in particular perennial grasses, is the greater potential for transgene escape given their ability to spread pollen over large distances and the large number of close relatives of the targeted commercial varieties and species used at this time. The threat of spreading herbicide resistance into weedy grass species is a real possibility and one that could have significant effects on agriculture and the environment.

Recently, H. Luo, A.P.K., J. Chandlee and M.O. (unpublished) proposed a mechanism to eliminate all possibility for gene transfer in species that are grown primarily for their green biomass, in particular turf grasses. The strategy is simply to prevent flower formation in plants that are released into the field. The mechanism makes use of a site-specific recombinase (in this case the FLP/FRT system from yeast) to activate a gene designed to down-regulate a critical gene in the initiation of floral development. The gene targeted for down-regulation is *FLORICAULA*/*LEAFY*, which regulates 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—and an 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 express FLP constitutively, 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 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.

11.2.8 Total Sterility and Confinement Expression Systems

Recently, research conducted by Ceres has described a new innovative total sterility confinement strategy. The Ceres Confinement & Expression System utilizes an "Activation" line as the male, which is comprised of a proprietary promoter–yeast transcription factor (T)®. The "Target" line is then the female and pollination is by self-incompatibility, hand pollination or other male sterility systems. An upstream activation sequence (UAS) uses a Ceres gene that inhibits flowering or causes sterility (CPG)® for example, –UAS-CPG_1, UAS-CPG_2,. . .UAS-CPG_n for introduction of stacked traits.

Advantages of the Ceres Expression & Confinement System include: (1) targeted gene expression dependent on the activation line; (2) multiple proteins can be driven by the same promoter without silencing; (3) transcription and protein level

of individual proteins can be modulated by the number of copies of the target gene and UAS elements in the gene; (4) achieves a three- to ten-fold amplification of expression relative to direct fusions; and (5) male and female sterility can be achieved in the commercial seed while allowing breeding to occur. The benefits of this type of program are that target proteins are produced only in the production field; no pollen is produced by plants expressing the target proteins; plants do not express the target proteins unless pollinated by the activation line; and pollen that leaves the production field will express only the transcription factor. Total sterility must be selectable and highly efficient for release as a commercial product.

11.3 Regulatory Issues for Perennial Bioenergy-Dedicated Crops

Currently, The USDA APHIS regulates release of GE plants on a case-by-case basis. The process of deregulation includes lengthy reviews and data collection spanning different environments and several years, with consideration of several factors including: the biology, geography and ecology of the plant: the trait gene(s) of interest; the possibility of gene flow to wild and non-transgenic relatives; the possibility of weediness or invasiveness; and unintended consequences to other organisms. It is important to assess independently the individual species of dedicated energy crops and their novel traits or characteristics that might enhance the vigor or invasiveness of wild or weedy relatives or have other detrimental effects. While some traits may pose relatively benign risk (i.e., herbicide tolerance) others may promote unintended consequences and invasiveness (i.e., drought and pest tolerance). Many of the dedicated energy crops that are currently considered to play a major role in the developing biofuels industry are perennial and have wild relatives in areas where they will be produced and grown. To date, there is no clearly defined limit to gene flow into the environment, which begs questions concerning acceptable (if any) levels of transgene escape in these plants; zero escape is a very stringent requirement. Considering the cost of deregulation and the subsequently imposed market restrictions, some regulatory requirements may be reconsidered or modified without compromising safety (Bradford et al. 2005). These might include: deregulation of the transgenic process itself, the 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.

11.4 Conclusions

Biotechnology approaches to genetic improvement of biofuels crops will undoubtedly play a large role in the development of a successful cellulosic energy industry. Certainly the development of regionally selected germplasm, marker-assisted

breeding and genomics will facilitate the selection of biofuels traits. In addition, the importance of transgenic traits will further accelerate progress towards the generation of dedicated energy varieties that will allow cost-effective low-input sustainable road fuels with lower greenhouse gas emissions. However, while numerous laboratories are currently exploring expression of transgenic plants for improved biofuels, the requirements for deregulation and commercialization of these crops remains uncertain. Robust gene confinement strategies must be in place as a part of biofuels trait modification. However, even with the best technologies in place it is unlikely that any of these will achieve a zero tolerance expectation. Therefore it seems reasonable to consider now, based on existing work in transgenic grasses, environmentally acceptable levels of mitigation.

We have discussed the available strategies for GE confinement that are currently under development. There are obvious limitations to most of these strategies, most notably, physical, spatial mechanical and temporal containment, but also some of the more sophisticated transgenic approaches that have not yet been developed for most dedicated energy crops and will need to be field tested. The use of genetic modification specifically for controlled transgenic containment is at an early stage of development and there are a range of possible approaches. Pollen sterility has been accomplished in a number of transgenic plant species but may be considered to be limited in its application for controlling gene flow because of the possibility of gene flow via seed scatter. It may be argued, however, that male sterility is sufficient for mitigating gene flow, as wild type crosses would produce progeny that would then also be male sterile, but this needs to be rigorously tested in the field. Also, 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 confinement and may also provide a valuable breeding tool. Maternal inheritance through plastid transformation is relatively well developed for some dicot plants; however, it may not offer complete containment, and has not been conferred widely on monocot crops. The GeneSafe technology and other seed-based GURTS offer conditional lethality that can be induced chemically to prevent flowering or seed development but requires complete biological induction and has 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 non-functioning 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 hybrid system whereby a two gene system is constructed such that, when crossed, the progeny will produce seed that will never again germinate and result in total sterility may offer the most promise for perennial dedicated energy crops. Also, it may be possible to include failsafe and backup mechanisms, including transgene mitigation strategies into a platform variety that can then receive stacked genes for crop improvement.

The potential benefits of GM of dedicated energy crops are obvious from the examples of food crops already in production. Moving forward, landscape-scale field testing and monitoring of genetic containment systems for perennial dedicated

energy crops must be accomplished to determine their efficacy. This should include guidelines established by regulatory agencies concerning acceptable levels of gene flow.

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