

REVIEW: PART OF A HIGHLIGHT ON BREEDING STRATEGIES
FOR FORAGE AND GRASS IMPROVEMENT

Is genetic engineering ever going to take off in forage, turf and bioenergy crop breeding?

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• **Background** Genetic engineering offers the opportunity to generate unique genetic variation that is either absent in the sexually compatible gene pool or has very low heritability. The generation of transgenic plants, coupled with breeding, has led to the production of widely used transgenic cultivars in several major cash crops, such as maize, soybean, cotton and canola. The process for regulatory approval of genetically engineered crops is slow and subject to extensive political interference. The situation in forage grasses and legumes is more complicated.

• **Scope** Most widely grown forage, turf and bioenergy species (e.g. tall fescue, perennial ryegrass, switchgrass, alfalfa, white clover) are highly self-incompatible and outcrossing. Compared with inbreeding species, they have a high potential to pass their genes to adjacent plants. A major biosafety concern in these species is pollen-mediated transgene flow. Because human consumption is indirect, risk assessment of transgenic forage, turf and bioenergy species has focused on their environmental or ecological impacts. Although significant progress has been made in genetic modification of these species, commercialization of transgenic cultivars is very limited because of the stringent and costly regulatory requirements. To date, the only transgenic forage crop deregulated in the US is 'Roundup Ready' (RR) alfalfa. The approval process for RR alfalfa was complicated, involving several rounds of regulation, deregulation and re-regulation. Nevertheless, commercialization of RR alfalfa is an important step forward in regulatory approval of a perennial outcrossing forage crop. As additional transgenic forage, turf and bioenergy crops are generated and tested, different strategies have been developed to meet regulatory requirements. Recent progress in risk assessment and deregulation of transgenic forage and turf species is summarized and discussed.

Key words: Forage and turf, bioenergy crops, grass, legume, transgenic plant, gene flow, deregulation, biosafety, transgenesis, alfalfa, creeping bentgrass, Kentucky bluegrass, Roundup Ready.

INTRODUCTION

Forage crops are critical to the livestock industry and sustainable agriculture worldwide. Commonly used forage crops include both monocotyledonous grasses and dicotyledonous legumes. They represent a diverse group of plants that have annual or perennial life cycles, cool- or warm-season growth preferences, and sod-forming or bunch-type growth habits. The widely cultivated forage grasses include tall fescue (*Festuca arundinacea*), perennial ryegrass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*), orchardgrass (*Dactylis glomerata*), Kentucky bluegrass (*Poa pratensis*) and bermudagrass (*Cynodon dactylon*). Major forage legumes include alfalfa (*Medicago sativa* L.) and white clover (*Trifolium repens*). Besides being used as forage crop, some slow growing, dwarf-type grasses are grown specifically for turf or amenity purposes on sports fields, lawns and roadsides. Turf species, such as tall fescue, perennial ryegrass, bermudagrass, Kentucky bluegrass and creeping bentgrass (*Agrostis stolonifera*), contribute considerably to our environment by

providing a safe playing surface for recreation and preventing erosion (Spangenberg *et al.*, 1998; Wang and Ge, 2006).

Some of the forage species are highly productive and have the potential to be used as bioenergy crops. Biofuels produced from lignocellulosic biomass are called lignocellulosic biofuels. Such biomass contains abundant sugars in the form of cellulose and hemicellulose, which can be converted to ethanol by hydrolysis and subsequent fermentation (Hisano *et al.*, 2009). A good example of a herbaceous biofuel crop is switchgrass (*Panicum virgatum*). Switchgrass is a C4 warm-season perennial species native throughout North America. It has been chosen as a model bioenergy species by the US Department of Energy because of its high biomass production, wide geographical distribution, high nutrient use efficiency and environmental benefits (McLaughlin and Kszos, 2005; Schmer *et al.*, 2008; Casler *et al.*, 2011).

Transgenic technology has been used to improve forage, turf and bioenergy crops. Because the technology allows the introduction of foreign genes from unrelated species and the down-regulation or upregulation of endogenous genes, it offers the

opportunity to introduce novel genetic variation into plant breeding programmes. Since the production of the first transgenic forage-type tall fescue plants (Wang *et al.*, 1992), tremendous progress has been made in genetic engineering of forage, turf and bioenergy crops in the last two decades. Some of the achievements in genetic engineering of grasses and legumes have been reviewed by Wang and Ge (2006) and Kölliker *et al.* (2010). In brief, transgenic approaches have been employed to improve these species in the following aspects: significant improvement of *in vitro* dry matter digestibility in alfalfa, tall fescue and perennial ryegrass (Guo *et al.*, 2001; Chen *et al.*, 2003, 2004; Reddy *et al.*, 2005; Tu *et al.*, 2010); enhanced drought tolerance in alfalfa, white clover, creeping bentgrass and bahiagrass (*Paspalum notatum* Flugge) (J.-Y. Zhang *et al.*, 2005, 2007; Fu *et al.*, 2007; Jiang *et al.*, 2009, 2010; Xiong *et al.*, 2010); increased phosphorus acquisition in white clover and alfalfa (Ma *et al.*, 2009, 2012); enhanced salt tolerance, cold tolerance or freezing tolerance in perennial ryegrass, tall fescue and creeping bentgrass (Hisano *et al.*, 2004; Hu *et al.*, 2005; Wu *et al.*, 2005; Li *et al.*, 2010); delay or inhibition of floral development in red fescue (*Festuca rubra*) (Jensen *et al.*, 2004); development of hypo-allergenic perennial and Italian ryegrasses (Petrovska *et al.*, 2004); enhanced aluminium tolerance in alfalfa (Tesfaye *et al.*, 2001; Barone *et al.*, 2008); delay of leaf senescence in alfalfa (Calderini *et al.*, 2007; C. Zhou *et al.*, 2011); virus-resistant perennial ryegrass and white clover (Xu *et al.*, 2001; Ludlow *et al.*, 2009); increased disease resistance in tall fescue and creeping bentgrass (Fu *et al.*, 2005; Dong *et al.*, 2007, 2008; M. Zhou *et al.*, 2011); improved turf quality in bahiagrass (Agharkar *et al.*, 2007; H. Zhang *et al.*, 2007); accumulation of sulphur-rich protein in subterranean clover (*Trifolium subterraneum* L.) and tall fescue (Rafiqul *et al.*, 1996; Wang *et al.*, 2001); production of polyhydroxybutyrate in switchgrass (Somleva *et al.*, 2008); increased sugar release in alfalfa and switchgrass (Chen and Dixon, 2007; Jackson *et al.*, 2008; Fu *et al.*, 2011a, b; Saathoff *et al.*, 2011); increased biomass yield in switchgrass (Fu *et al.*, 2012); and a large improvement in bioethanol production in switchgrass (Fu *et al.*, 2011a).

Genetic engineering has greatly contributed to breakthroughs in plant improvement and led to the development of widely grown cultivars in major cash crops (Park *et al.*, 2011). The adoption of transgenic crops in the last 15 years has experienced an 87-fold increase since biotech crops were first commercialized in 1996, making biotech crops the fastest adopted crop technology in history. The accumulated growth areas from 1996 to 2010 exceeded 1 billion hectares (James, 2011). The number of countries planting biotech crops reached 29 in 2010 and the top ten countries each grew more than 1 million hectares. The United States remains the biggest adopter of transgenic crops, with 66.8 million hectares planted in 2010, which represent 45% of the global biotech area (James, 2011).

Despite the wide adoption and the beneficial economic and environmental impacts of transgenic crops, it has been extremely difficult to deregulate and commercialize new transgenic cultivars. The situation is even more complicated in transgenic forage, turf and bioenergy species. One enduring lesson from agricultural biotech is that it is a huge

mistake to underestimate biosafety concerns (Stewart, 2007). In this paper, we focus our discussions on the deregulation process of transgenics in the US only. Specific successful and unsuccessful examples will be given to illustrate the process and the complications involved in deregulation of forage and turf.

REGULATORY SYSTEMS AND COMPLICATIONS IN COMMERCIALIZATION OF TRANSGENIC CROPS

In the US, the federal agencies involved in assessing transgenic plants or genetically modified organisms (GMOs) are the US Department of Agriculture (USDA), the Environmental Protection Agency (EPA) and the Food and Drug Administration (FDA). Most developed genetically modified plants are reviewed by at least two of these agencies, with many subject to all three (McHughen and Smyth, 2008).

The Animal and Plant Health Inspection Service (APHIS) is the regulatory branch of the USDA. It is primarily concerned with protecting agriculture and the environment from potential pests. APHIS determines whether a transgenic cultivar is likely to become a pest, i.e. to have negative agricultural or environmental effects. The agency regulates the import, transportation and field test of transgenic plants and seeds through notification and permitting procedures.

The EPA regulates transgenic plants that are engineered for pest resistance, for example Bt insect-resistant or virus-resistant plants. In EPA terminology, these plants contain 'plant-incorporated protectants' (also known as 'plant-pesticides'). The EPA claims not to regulate genetically engineered (GE) plants *per se*, but rather it regulates the pesticidal properties associated with a GE plant (McHughen and Smyth, 2008).

The FDA is responsible for ensuring the safety of human food and the supply of animal feed. If the introduced gene is from a known allergenic source, then the transgenic food must be assessed for allergenicity. If foods or feeds produced from or with GMOs are composed of the same substances and in the same amounts and relative proportions, then there is no basis for a safety concern and no need to invoke the 'adulteration' action trigger (McHughen and Smyth, 2008). In contrast to most other regulatory agencies worldwide, which trigger regulatory scrutiny based on the mere process of genetic engineering, the FDA regulates foods and feeds based on the objective changes in product composition. The FDA is almost unique in having a scientifically sound basis for its regulatory trigger: recognizing that hazard is caused by the presence of tangible substances, not by the breeding method (McHughen, 2007; McHughen and Smyth, 2008).

Although the 148 million hectares of transgenic crops in 2010 occupied a significant 10% of all 1.5 billion hectares of cropland in the world (James, 2011), a detailed look at the data revealed that more than 99% of the biotech areas are occupied by transgenic maize, soybean, cotton and canola. Besides these four major cash crops, only a few other species have been deregulated, including papaya, squash, rice, potato and alfalfa. Regarding transgenic traits, herbicide tolerance has consistently been the dominant trait, followed by insect resistance and virus resistance.

The very limited number of transgenic crops deregulated and the few traits used in commercial plantings are in sharp contrast to the large number of successful genetic engineering reports available in the literature. The deregulation process has become so complicated and costly that only large companies with deep resources can afford to do it. As pointed out by Bradford *et al.* (2005), the costs of meeting regulatory requirements and market restrictions guided by regulatory criteria are substantial impediments to the commercialization of transgenic crops (Bradford *et al.*, 2005). Obviously, this creates big problems for transgenic improvement and commercialization of specialty crops and specific traits.

Most of the important forage, turf and bioenergy species are outcrossing and have perennial growth habit. These plants can readily cross with wild or feral relatives. Some species have good adaptation to marginal land and possess invasive capabilities. Regulatory agencies have raised special concerns and required additional scrutiny for these perennial grasses and legumes (Strauss *et al.*, 2010). A major concern is pollen-mediated transgene flow.

GENE FLOW STUDIES IN FORAGE AND TURF

Pollen is an important vector of gene flow in outcrossing species. A simple pollen germination medium was used to assess *in vitro* viability and longevity of tall fescue and switchgrass pollen (Wang *et al.*, 2004a; Ge *et al.*, 2011). Weather conditions have a large impact on pollen longevity. Under sunny atmospheric conditions, viability of transgenic and non-transgenic tall fescue pollen declined to 5% in 30 min, with a complete loss of viability in 90 min. Under cloudy atmospheric conditions, viability of tall fescue pollen declined to 5% after 150 min, with a complete loss of viability in 240 min (Wang *et al.*, 2004a). Similarly, switchgrass pollen longevity decreased rapidly under sunny atmospheric conditions, with a half-life of less than 4.9 min and a complete loss of viability in 20 min. Under cloudy atmospheric conditions, the half-life of switchgrass pollen was more than five-fold longer than under sunny conditions, and it took approx. 150 min to lose viability completely (Ge *et al.*, 2011). In both tall fescue and switchgrass, no difference in pollen viability and longevity was found between transgenic and non-transgenic control plants.

As wind-pollinated grasses have a high potential to pass their genes to adjacent plants, pollen flow is not only a concern in transgenics; it has long been a consideration for seed purity of conventional cultivars. For foundation seed production of cross-pollinated grasses, the isolation standard required by the USDA (CFR201.76) is 274 m (900 feet) isolation distance. Field trials of transgenic grasses have basically followed the standard for foundation seed production. In the case of field release of transgenic forage, turf and bioenergy crops, human consumption is indirect or not involved at all. Thus, evaluation of the biosafety of these species mainly focuses on their environmental or ecological impacts.

Morphological markers were first used to study pollen flow several decades ago. Pollen contamination in perennial ryegrass by an outside pollen source was reported to be as much as 10% at 182 m (Griffiths, 1951) or as little as 1% at 7.3 m (Copeland and Harding, 1970). In smooth bromegrass

(*Bromus inermis*), Johnson *et al.* (1996) reported pollen contamination of less than 5% between 22 and 27 m (Johnson *et al.*, 1996). A pollen trap study in perennial ryegrass showed that ryegrass pollen could travel 80 m, although the amount of pollen collected at this distance was much less than in the traps near the centre ryegrass field (Giddings *et al.*, 1997a, b). An isozyme marker (*Pgi-2*) was used to investigate pollen dispersal in meadow fescue (*Festuca pratensis* Huds.); pollen was captured by recipient plants at a distance of 250 m (Nurminiemi *et al.*, 1998; Rogli *et al.*, 2000).

Transgenic plants provide unique materials to study gene flow in grasses. In a small-scale field trial of transgenic creeping bentgrass, herbicide-resistant progenies were recovered at 292 m maximum distance from the source transgenic plants (Wipff and Fricker, 2001). Frequencies of interspecific hybridization between transgenic creeping bentgrass and four related species were measured, with interspecific transgenic hybrids recovered between creeping bentgrass and *Agrostis capillaris* and *A. castellana* at frequencies of 0.044 and 0.0015%, respectively (Belanger *et al.*, 2003). In a small-scale experiment on pollen dispersal of transgenic tall fescue, transgenes were detected in recipient plants at up to 150 m from the central transgenic plot. The highest transgene frequencies, 5% at 50 m, 4.12% at 100 m and 0.96% at 150 m, were observed in the prevailing wind direction (Wang *et al.*, 2004b). Assessment of cross-pollination in zoysiagrass (*Zoysia japonica* Steud.) revealed that gene flow from transgenic zoysiagrass was not detected in neighbouring weed species examined, but was observed in wild-type zoysiagrass with a frequency of 1.2% at a distance of 0.5 m, 0.12% at a distance of 3 m and 0% at distances over 3 m (Bae *et al.*, 2008).

Apomixis is known to exist in many warm-season grasses, such as *Poa* and *Paspalum* species. Apomictic reproduction mode is characterized by embryo development, which is independent of fertilization of the egg cell, but requires fertilization with compatible pollen to produce the endosperm (Sandhu *et al.*, 2010). Transgenic Kentucky bluegrass was used as a pollen donor to quantify intra- and interspecific pollen-mediated gene flow. Twenty-five sexual and facultative apomictic *Poa* species were used as pollen receptor and placed at 0, 13 and 53 m distances from the transgenic materials. Overall hybrid frequency was 0.048% and hybrid frequency at the 0-m distance was 0.53% (Johnson *et al.*, 2006). To quantify gene flow from apomictic tetraploid bahiagrass (*Paspalum notatum* Flugge) to tetraploid or diploid bahiagrass, the glufosinate-resistant apomictic bahiagrass was grown at close proximity (0.5–3.5 m) with non-transgenic cultivars. Average gene transfer between transgenic apomictic, tetraploid and sexual diploid bahiagrass was 0.03%. Average gene transfer between transgenic apomictic tetraploid and non-transgenic, apomictic tetraploid bahiagrass was 0.17% (Sandhu *et al.*, 2010). While not providing complete transgene containment, gene transfer between apomictic species occurs at low frequency and over short distances (Johnson *et al.*, 2006; Sandhu *et al.*, 2010).

In a landscape-level of study for 'Roundup Ready' creeping bentgrass, it was found that most of the gene flow occurred within 2 km in the direction of prevailing winds. The maximal gene flow distances observed were 21 and 14 km in sentinel and resident plants, respectively, that were located in

primarily non-agronomic habitats (Watrud *et al.*, 2004). This report gained a considerable amount of publicity and played a role in tightening the regulatory process of APHIS on outcrossing grasses. Prior to 2005, field trials of outcrossing transgenic grasses required only a simple notification to APHIS. Since 2005, field release of such species has been tightened, requiring a full permit. The procedures and requirements are more stringent if the transgenic grasses are allowed to flower in the field.

Alfalfa and white clover are predominantly pollinated by insects. A large-scale field study of 'Roundup Ready' alfalfa showed that pollen-mediated gene flow diminished with increasing distance from the source. Gene flow among the worst-case management plots was 1.39, 0.32, 0.07 and 0.003 % at 152, 305, 457 and 805 m, respectively. No gene flow was detected at 1.6 km (Fitzpatrick *et al.*, 2003). Another study using 'Roundup Ready' alfalfa showed that honey-bee-mediated gene flow was 1.49 % at 274 m and it decreased linearly to 0.20 % near 1524 m. Gene flow continued to decline out to 4.1 km where it was detected at a low frequency (<0.06 %) (Teuber *et al.*, 2004).

Gene flow is a natural event that happens all the time, but the introduction of modern biotechnology has brought new attention to this natural process and raised ecological, economic as well as intellectual property issues for scientists and policymakers to consider. A main focus in risk assessment research should be placed on the consequences of transgene flow. The phenotypes of transgenic plants and their safety in the environment, not the method used to produce them, should be the main focus of risk analyses and regulatory concern (Bradford *et al.*, 2005).

DEREGULATION OF 'ROUNDUP READY' ALFALFA

Although significant progress has been made in the genetic engineering of forage, turf and bioenergy species, to date, the only deregulated crop is 'Roundup Ready' alfalfa. The deregulation process is lengthy and complicated.

In April 2004, USDA-APHIS received a petition from Monsanto and Forage Genetics International requesting a determination of non-regulated status of alfalfa lines tolerant to the herbicide glyphosate, the active ingredient in the herbicide Roundup®. The trait was obtained by transgenic expression of the 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) gene. The gene was derived from the CP4 strain of *Agrobacterium tumefaciens*. The transgenic alfalfa plants were obtained by *Agrobacterium*-mediated transformation. The non-selective herbicide glyphosate inhibits an essential step in aromatic amine synthesis in plants by blocking the action of the natural EPSPS enzymes already present in the plant. However, the CP4 EPSPS protein is not inhibited by glyphosate; thus any plant expressing sufficient levels of this protein is tolerant to glyphosate application. The 'Roundup Ready' alfalfa is also called glyphosate-tolerant (GT) alfalfa.

APHIS assessed the plant pest risks posed by the use of transgenic alfalfa and prepared an Environmental Assessment. Effective from 14 June 2005, APHIS deregulated the GT alfalfa.

Approximately nine months later, a group of organic alfalfa growers and the Center for Food Safety filed a lawsuit in the Northern District of California that challenged APHIS' decision to grant non-regulated status to the alfalfa lines. In February 2007, the court ruled that APHIS' Environmental Assessment failed to adequately consider certain environmental and economic impacts, and the court overruled APHIS' decision to grant non-regulated status to transgenic alfalfa. The court also ordered the USDA to prepare an Environmental Impact Statement (EIS), a lengthy and complicated document requiring careful deliberation and wide collaborative effort. Until this recent spat involving alfalfa in the US courts, no transgenic crop approved by the USDA had required an EIS in the approval process (Waltz, 2011b). New plantings of transgenic alfalfa were halted. The existing 200 000 total acres were still allowed to be harvested, used and sold, but the fields were subject to court-ordered stewardship practices to minimize the potential of transgenic alfalfa's presence in harvests of non-transgenic alfalfa.

The 'Roundup Ready' alfalfa case went to the US Supreme Court. In June 2010, the Supreme Court issued a ruling on this matter in favour of Monsanto, saying that the district Court had overreached itself procedurally in halting the plantings. The ruling allowed the USDA-APHIS to take appropriate action to allow further planting while they completed the EIS.

APHIS released a draft EIS in December 2009 and received approx. 244 000 public comments. APHIS produced a final EIS on 16 December 2010. The 2300-page document concludes that the transgenic alfalfa is safe for food and feed purposes and is unlikely to pose plant pest risks. The agency at first proposed one of two actions: either to approve the transgenic alfalfa fully or approve the crop in part, with restrictions on isolation distances and geographical locations. The partial approval proposal caused serious concerns from the biotech industry, because such a move would set a precedent where commercial motives would prevail over science-based decisions from the USDA. Farm groups claimed that their international trade efforts would be undermined if the USDA moved forward with injecting non-science-based criteria into the regulatory process. In a letter to the USDA sent by several US congressmen, they called the proposal 'disturbing' because it 'politicizes the regulatory process' (Waltz, 2011b).

On 27 January 2011, the USDA announced it would fully deregulate alfalfa without restrictions. After a 4-year court-imposed ban, US farmers can again grow GE alfalfa.

'ROUNDUP READY' CREEPING BENTGRASS

Creeping bentgrass is a slow-growing grass species exclusively used for turf purposes. Transgenic creeping bentgrass was developed by introducing the CP4 EPSPS gene using a biolistic device (microprojectile bombardment). The gene insertion allows the use of glyphosate as a weed control option in turf-grass production. If the creeping bentgrass plants producing CP4 EPSPS are growing in golf courses with weeds, applying glyphosate will kill the weeds but not the bentgrass plants.

APHIS received a revised petition in April 2003 from the Scotts Company and Monsanto Company seeking a determination of non-regulated status for a creeping bentgrass that is genetically engineered to be tolerant to the herbicide

glyphosate. 'Roundup Ready' creeping bentgrass was the first perennial biotech grass plant reviewed by APHIS.

In 2002, the Scotts Company planted approx. 160 ha of 'Roundup Ready' creeping bentgrass in central Oregon under USDA-APHIS permit. When the source fields of creeping bentgrass flowered for the first time during summer 2003, the CP4 EPSPS gene was used as a marker to quantify viable GM pollen movement and potential gene flow to compatible resident and sentinel plants. It was found that hybridization of *Agrostis* plants by viable transgenic pollen happened as far as 21 km beyond the perimeter of the bentgrass source area (Watrud *et al.*, 2004).

In 2003, APHIS completed a weed risk assessment and determined that GE glyphosate-tolerant creeping bentgrass did not meet the criteria to be regulated as a federal noxious weed. The Center for Food Safety challenged APHIS' decision in federal court. On 5 February 2007, a judge of the US District Court of Washington issued a ruling that for the most part favoured several environmental groups. Citing the 2004 study (Watrud *et al.*, 2004), the judge agreed with the plaintiffs that the risk of engineered grasses becoming established in protected grasslands was 'non-trivial'. He also concluded that some of the positions taken by USDA officials regarding their evaluation of potentially noxious weeds were 'arbitrary and capricious and contrary to law', and thus he ordered that further field testing await a full environmental assessment by the USDA under the National Environmental Policy Act (NEPA). The USDA did not appeal the decision and instead instituted new NEPA policies for any future field tests. The Scotts Company appealed the decision. In March 2008, the Federal Court of Appeals dismissed the case.

Obviously, an EIS is necessary for transgenic creeping bentgrass. A draft EIS has not been completed or released by APHIS.

HERBICIDE-TOLERANT KENTUCKY BLUEGRASS

The Scotts Company produced new glyphosate-tolerant transgenic Kentucky bluegrass without using plant pest components. Specifically, the transgenic plants were produced by biolistic transformation, without involving *Agrobacterium* transformation or any other plant pest regulated under the Plant Protection Act. The herbicide resistance gene EPSPS is from *Arabidopsis thaliana*, the ubiquitin promoter is from rice, the actin intron is from rice and the alcohol dehydrogenase 3' untranslated region is from maize.

APHIS defines a 'regulated article' as: any organism which has been altered or produced through genetic engineering, if the donor organism, recipient organism, or vector or vector agent belongs to any genera or taxa designated in § 340.2 and meets the definition of plant pest, or is an unclassified organism and/or an organism whose classification is unknown, or any product which contains such an organism, or any other organism or product altered or produced through genetic engineering which the administrator determines is a plant pest or has reason to believe is a plant pest.

In other words, if a GE organism is not a plant pest, is not made using plant pests and APHIS has no reason to believe

that it is a plant pest, then the GE organism would not fall under APHIS' regulatory authority.

APHIS has determined that Kentucky bluegrass, whether GE or not, should not be listed as a noxious weed. In July 2011, APHIS confirmed that the transgenic bluegrass produced by Scotts is not within the agency's regulatory authority because it does not contain plant pest sequences and no plant pest was used to create the GE Kentucky bluegrass. The glyphosate tolerance is caused by a single gene insertion, which does not create a new species of Kentucky bluegrass, meaning it is biologically the same as its traditional counterpart (USDA-APHIS, 2011). Essentially, the GE Kentucky bluegrass is not a 'regulated article' for purposes of the Plant Protection Act.

The decision that transgenic Kentucky bluegrass is not a regulated article is consistent with other APHIS regulatory determinations. For example, in 2008, APHIS concluded that GE petunias that were transformed using genes derived from *Petunia hybrid* and *Escherichia coli*, and transferred by biolistics were not regulated articles. Although the idea of bypassing USDA review by going through the non-plant-pest route has been known to industry players for a long time, it seems the large biotech companies have no interest in testing the system while enjoying the present complicated deregulatory process, which automatically prevents the public sectors and small companies from getting involved due to the cost and time required (Waltz, 2011a).

INTRAGENESIS AND CISGENESIS

One of the public concerns about transgenic crops relates to the mingling of genetic materials among distantly related organisms. New molecular strategies have been designed to address the issue. Intragenesis (Rommens *et al.*, 2004, 2007) or cisgenesis (Schouten *et al.*, 2006a, b) refers to the introduction of one or more genes that are derived from the target species itself or species that are sexually compatible with the target species. Cisgenesis is more restrictive in that it refers to the transfer of a complete DNA copy of a natural gene, including its promoter and terminator (Schouten and Jacobsen, 2008). It is obvious that intragenic or cisgenic plants are closer to their natural counterparts than the above-mentioned Kentucky blue grass.

Conventional breeding employs methods such as introgression and mutagenesis to modify a plant genome randomly and, as a result, create genetic variation (Rommens *et al.*, 2007). In the case of intragenic or cisgenic plants, the gene of interest, together with its regulatory sequences, has been present in the species or in a sexually compatible relative for centuries (Schouten *et al.*, 2006a). Therefore, the gene pool exploited by intragenesis and cisgenesis is identical to the gene pool available for traditional breeding (Holme *et al.*, 2012). Furthermore, no changes in fitness occur that would not happen through either conventional breeding or natural gene flow. Intragenic or cisgenic plants carry no additional risks – such as effects on non-target organisms or soil ecosystems, toxicity or a possible allergy risk for GM food or feed – other than those that are also incurred by conventional breeding (Schouten *et al.*, 2006a).

By avoiding the transfer of foreign or unknown DNA, crops developed through intragenesis or cisgenesis mimic the conventionally bred cultivars. In fact, they have much less gene shuffling than the conventional cultivars. By eliminating various potential risk factors, the intragenic or cisgenic method represents a relatively safe approach to crop improvement (Rommens *et al.*, 2007). Therefore, it has been argued that intragenic or cisgenic plants should be treated as conventionally bred plants (Schouten *et al.*, 2006a; Rommens *et al.*, 2007). Considering gene flow and other biosafety issues in forage, turf and bioenergy crops, the intragenic or cisgenic approach may provide a cost-effective way for genetic engineering of these species.

TRANSGENE CONTAINMENT

A number of biological containment measures have been developed or proposed to control transgene flow, including male sterility, seed sterility, maternal inheritance, delayed flowering and gene-deletor (Daniell, 2002; Luo *et al.*, 2007; Clarke and Daniell, 2011).

Male sterility is very effective at preventing outcrossing from transgenic plants to weeds or related non-GM species. The technology has been commercially exploited in canola. Selective ablation of tapetal cells by cell-specific expression of nuclear genes encoding cytotoxic molecules or an antisense gene essential for blocking pollen development give rise to stable male sterility (Kausch *et al.*, 2009). In creeping bentgrass, 100% pollen sterility was obtained by expressing a ribonuclease gene, barnase, under control of a rice tapetum-specific promoter (Luo *et al.*, 2005).

Another effective way of preventing pollen-mediated gene flow is chloroplast engineering. Expression of foreign genes in chloroplasts could confine pollen transmission because of the maternal inheritance of chloroplasts (Hagemann, 2010). Besides transgene containment, chloroplast genetic engineering offers other advantages, such as high level production of foreign proteins, a high-precision site-specific transgene integration exclusively via homologous recombination, the absence from plastids of epigenetic effects and gene silencing mechanisms, and the lack of pleiotropic effects due to subcellular compartmentalization (Clarke and Daniell, 2011). Although transplastomic plants have been obtained in a number of species, such as lettuce, tomato, potato, poplar, carrot, cotton and soybean, there have been no reports on successful chloroplast transformation in forage, turf or bioenergy grasses. With recent developments in grass tissue culture and regeneration, it is foreseeable that effective chloroplast transformation systems will be developed for these species in the near future. Although organelle genes are in general maternally inherited, there are rare exceptions (Daniell, 2007). For example, biparental transmission of plastids has been shown to occur in alfalfa (Lee *et al.*, 1988; Masoud *et al.*, 1990). Obviously, transgene containment via maternal inheritance would not be applicable to the plant species that show biparental inheritance of chloroplast genomes.

Delayed flowering and complete floral inhibition have been considered desirable target traits for genetic manipulation of grasses (Wang and Ge, 2006). Delay of flowering will minimize the risk of cross pollination between transgenic and wild-

type plants. Inhibition of flowering will completely avoid the problem of transgene flow in grasses. An additional benefit is that inhibition of flowering will reduce allergic reactions caused by grass pollen, a major source of allergenic protein (Petrovska *et al.*, 2004). Inhibition of floral development was obtained in red fescue by transgenically expressing a strong floral repressor, TERMINAL FLOWER1 (LpTFL1), isolated from perennial ryegrass (Jensen *et al.*, 2004). Recently, it has been demonstrated that overexpression of a microRNA (miR156) in switchgrass led to the production of non-flowering transgenic plants (Fu *et al.*, 2012). The systems have the potential to be directly used in grasses that are vegetatively propagated commercially. For seed-propagated species, future study should involve the development of a seed production system.

CONCLUDING REMARKS

Plant improvement is needed to enhance our ability to produce food, feed, fibre and fuel and to ensure we have a safe, liveable environment. Ideally, our plant improvement efforts would be done in a way that is in harmony with the environment (Brummer *et al.*, 2011). In addition to their main product or function, many forage, turf and bioenergy species have positive effects on farming systems and on the environment. For example, including forage crops, such as alfalfa, into a crop rotation with corn and soybean had both environmental and economic benefits (Olmstead and Brummer, 2008). The modern plant improvement technologies developed for genetic manipulation of various forage, turf and bioenergy species have opened up new opportunities for breeding these crops, which may help make them more valuable to cropping systems and hence more likely to become a component of them, bringing along their multifunctional benefits. Transgenesis, including nuclear transformation as well as intragenesis, cisgenesis and chloroplast transformation, provides a rapid means for plant improvement, and should be among the technologies being used as we attempt to develop improved crops to be included into sustainable cropping or landscape systems (Ronald, 2011).

The major challenge now is how to apply the technology to generate new genetic variability in a way that satisfies regulatory requirements. The development of an EIS for alfalfa and the deregulation of herbicide-tolerant alfalfa paved the way for future transgenic improvement of this important forage legume crop. For grasses, the development of intragenic or cisgenic lines is likely to be the first practical step toward deregulation.

We suggest three aspects that need to be considered in the regulatory process, both generally for all transgenic crops and specifically for the forage, turf and bioenergy species considered here.

- (1) Regulation of transgenics should be based on the risks posed by the features of the product, not the process of breeding. No evidence exists suggesting that the technologies *per se* involved in the development of transgenic crops pose a threat either to human health or to the environment. In a *New York Times* editorial of 18 August 2011, Nina Federoff makes the following appeal: 'It is time to relieve the regulatory burden slowing down the development of genetically modified crops. The three

United States regulatory agencies need to develop a single set of requirements and focus solely on the hazards – if any – posed by new traits.’ (Federoff, 2011). We support the call for streamlining the process and focusing on traits, not the technologies.

- (2) Gene flow within and between populations is an essential feature of outcrossing species such as many forage, turf and bioenergy crops because of their natural self-incompatibility. Therefore, a major focus in risk assessment research on these species should be placed on the consequences of transgene flow. That genes will flow from one population to another is obvious; the important aspect is what effect this gene flow will have economically or on human or ecosystem health. The risks need to be clearly obvious, and not hypothetically postulated. In addition to the risks posed by the transgene, similar assessments need to be made of the lost opportunities if the specific transgene is not deregulated.
- (3) Forage, turf and bioenergy species do not enter the food chain directly, or in some cases at all, and the regulatory hurdle needs to reflect this lower risk situation.

Despite various concerns, major transgenic crops have been widely cultivated and intensively consumed in the last 16 years with no documented cases of adverse effects on health or the environment. A streamlined regulatory system, designed to catch obvious hazards but not prevent entry into the marketplace by small companies and non-profit organizations, needs to be developed. By efficient incorporation of novel germplasm into applied breeding programmes, transgenic cultivars have the potential to play a critical role in fulfilling the increasing demand for animal products and renewable fuels in the 21st century, and in conjunction with ecologically driven farming practices, leading to an economically and environmentally sustainable agricultural system.

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