

The Science of Gene Flow in Agriculture and Its Role in Co-existence

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Meeting Report

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Background and Introduction

A conference on “**The Science of Gene Flow in Agriculture and Its Role in Co-existence**” was held at USDA in Washington, DC on September 7-8th, 2011. The goal of the conference was to have scientific leaders in the field, agricultural industry groups, farmers, governmental regulators and non-governmental groups discuss the impacts of gene flow on agriculture, current knowledge of gene flow and persistence of novel genes in the environment, and current and potential mitigation strategies for gene containment. This report synthesizes key presentations and discussions at the conference.

The location was chosen to facilitate participation by government agencies on this important topic. The meeting budget included full conference costs (i.e., no registration fees were charged) for 150 participants including speakers as well as any additional government employees. Grants were provided by the USDA Office of the Secretary and the National Institute for Food and Agriculture (NIFA) to support the conference. The conference was organized and administered by the Seed Biotechnology Center of the University of California, Davis. The conference was well advertised using electronic listserves for media coverage and to over 3000 people representing organic, conventional and biotechnology organizations. The conference was further advertised within government agencies. The conference was reposted in several media venues electronically and as hardcopy. One week prior to the conference, 150 participants had registered. Table 1 summarizes the affiliations of those who attended, including governmental personnel. Seven participants were international. Forty-three pre-registered participants did not attend. Of these, 23 were from government agencies and nine were from the organic industry or NGOs. At least four additional government personnel attended selected presentations. Based on these statistics, we believe that the goal of attracting a balanced and representative cross-section of the agriculture industry to the conference was achieved.

The program (see Appendix 1) consisted of 19 scientists from public institutions, associations, private industry and government agencies. Dr. Catherine Woteki, chief scientist for the USDA and Undersecretary for Research, Education and Economics, opened the conference with introductory remarks. The format featured 25-minute talks by each speaker and 30-minute panel discussions based on questions from the audience for each of three topic areas. This was followed by a 50-minute closing discussion on all three topics. The moderated discussion sessions were recorded and transcribed. The format allowed for several questions on each topic from the diverse audience and for discussion among experts.

Table 1. Distribution of attendees at Gene Flow in Agriculture conference, September 7-8th, 2011

Affiliation	Total
Association	11
Government	43
Industry	34
Organic industry*	11
Public/Academic	23
Total	122

*represents non-government associations associated with organic production and growers.

The sections that follow are organized into themes essentially as they were in the program. The report does not attempt to summarize or capture all information provided by each speaker, but rather to summarize the key points of both the presentations and panel discussions. Proceedings from the meeting (extended abstracts of each presentation) are posted at <http://sbc.ucdavis.edu/files2/geneflowcompleteproceedings2011.pdf>.

Managing Gene Flow in Agriculture

In the natural world, the ebb and flow of genes among individuals of a species is an important process that endlessly reshuffles genetic diversity. Gene flow between individuals within and among populations via pollen occurs only when they have concurrent geography, overlapping flowering times and share common pollinators. Given a sufficient population size (to avoid genetic drift), alleles that have no impact on fitness (positive or negative) will persist in the population at an allelic frequency equal to their introduction level. Alleles for genes conferring a fitness effect will be selected naturally for or against depending on the selection pressure. For example, the frequency of alleles conferring disease resistance may increase in the population in generations where a certain pathogen is prevalent (but not when it is absent), while alleles conferring herbicide tolerance will neither increase nor decrease in the population in areas where the herbicide is not used (Brule-Babel et al., 2006). Favorable genotypes for a certain trait are usually fixed at a more rapid rate in selfing than in outcrossing species. Genetic and biological features such as polyploidy, fecundity, and generation time also affect shifts in allele frequencies.

Genetic diversity is essential to make progress in plant breeding. Allele frequencies are shifted for traits of economic value, such as yield, quality, or seed shattering, and also for adaptation to environmental stresses or for disease and insect resistance. Plant breeders and even early farmers have sought genetic diversity conferred by gene flow among and within populations, only subsequently purifying genetic selections by inbreeding and fixing traits to reproduce economically favorable lines or cultivars. In the latter process, both physical and genetic (e.g., male sterility) mechanisms have been developed to stabilize and reproduce a desirable variety (e.g., a specific genetic composition) for farmers to grow. Thus, both gene flow and lack of gene flow are essential for agriculture.

The Importance of Understanding Gene Flow

On a scientific basis, gene flow can be defined as the transfer and introgression of genetic material (genes in living plant materials) from one plant to another. Gene flow is a two-way process that is ubiquitous in both natural and agricultural systems, but the extent to which it occurs depends on many factors, as do the consequences. In the context of trade, gene flow is used in a broader sense as the simple transfer of genes via seed or pollen dispersal where plant parts of different genotypes would be present in a population, even without genetic introgression. It can also include persistence in the environment through vegetative propagation. The terms adventitious presence (AP) or low level presence (LLP) are used in the industry to represent this type of gene flow. It can be neutral in effect or can have economic and biological consequences. Negative consequences in agriculture are largely associated with the unintended presence of certain genes or traits in products that require high genetic purity, such as in seed production or markets that restrict the presence of genetically engineered (GE) traits. Negative consequences in the environment could be associated with transfer of specific traits to related wild relatives, thereby altering their fitness or success relative to other plants.

Agriculture in the United States is a major supplier of many commodity and specialty crops in domestic and export markets and it depends on innovation to maintain its competitiveness in the global market. Consequently, to capture value, a diverse set of production and distribution systems must co-exist and meet specific market demands. At least three production systems, conventional, organic and biotech (using GE or transgenic crops), are used across the United States. These systems employ different product purity standards, and both domestic and

international commodity handling systems can maintain different standards even for the same products. Since agriculture involves biological systems and production in open environments, it is difficult to achieve 100% purity or 0% “contamination”. The factors affecting the level of intermixing of genetic materials through gene flow via pollen, seed, admixtures and vegetative propagation must be understood to develop practical non-zero thresholds and realistic market standards. Furthermore, as novel agricultural products are developed, an understanding of their potential for spreading and persisting in specific environments or in wild populations and the consequences thereof is needed prior to release.

Until recently, gene flow has primarily been a concern for the seed industry, which has developed certification programs and quality standards to assure buyers of the genetic purity of its products. Currently, controversial aspects of gene flow in agriculture largely derive from concerns about the possibility of genes from GE crops moving to related wild relatives or to conventional or organic crops. In some instances, large economic losses have occurred due to zero tolerances for admixtures due to gene flow, none of which were a safety concern. Nonetheless, in order to avoid market impacts and associated economic losses, a comprehensive understanding and control of gene flow as well as realistic thresholds are required for consistent marketing of agricultural commodities and organics.

The Seed Industry Model

The seed industry in the U.S., for instance, provides a time-tested model for practical segregation and identity preservation strategies. Co-existence between seed growers and farmers not growing seed relies on mutual respect and cooperation, a clear understanding of the biological restrictions of crop production systems and diligence and effort on the part of the seed grower to achieve required standards. Growers and the seed industry have developed these principles over the last 100 years, adjusting them as new information becomes available and as cropping systems evolve. For example, the Association of Official Seed Certifying Agencies (AOSCA) develops, monitors and coordinates standards for seed purity among 70 member countries. Similarly, the American Seed Trade Association (>800 members) works with the global seed industry to ensure that practical standards are developed to support market standards. Under current regulations, USDA/APHIS evaluates potential risks of biotech-derived crops to agriculture and the environment and the National Organic Program has set standards for materials and processes that may be used to produce certified organic products.

ASTA has recently released a practical guide to co-existence (American Seed Trade Association, 2011a) and seed production practices (American Seed Trade Association, 2011b). Current mitigation strategies are crop-specific, adjusting for the biology of the crop and the environment in which it is grown. For example, outcrossing rate, pollen type, pollinators, sexual compatibility, presence of related species, seed dispersal, fecundity and dormancy are considered when evaluating risk. Based on these, appropriate distances from compatible plants can be recommended for a desired level of purity. Crop rotations and specific handling techniques are also proposed. One of the most important components in all identity-preserved production systems is to begin with certified seeds that meet high genetic and physical purity standards. Seed purity tests (varietal purity, weed seeds, and inert matter) as well as genetic tests are used to monitor the effectiveness of the co-existence and identity-preservation standards. The ASTA, AOSCA and Association of Official Seed Analysts (AOSA) continuously work with the industry to update criteria to address evolving market and agricultural standards.

Crop considerations

Gene flow among plants has been reviewed recently (Gealy et al., 2007; Kwit et al., 2011). The potential for gene flow to wild relatives in the top 25 crops in the world have been reviewed (see Gealy et al., 2007, Appendix 2). Sexually compatible wild relatives exist in the US for cassava, cotton, grape, oats, oilseed rape, sorghum, sugarcane, sunflower and wheat. Examples of gene flow from transgenics to wild or weedy relatives have been reported in 13 species. Although hybridization has been shown in the species, introgression was studied only in Brassica, wheat and creeping bentgrass. In those cases, none of the weedy relatives indicated signs of invasiveness or selective advantage due to herbicide or insect resistance (Kwit et al., 2011, Appendix 3). A summary of gene flow studies was reviewed by Chandler and Dunwell (2008, see Appendix 4). Based on these, appropriate schemes can be recommended for a level of purity desired (see Appendix 5 and Seed Industry Model).

Self-pollinating species

Self-pollinating plants have flower structures that promote self-pollination, as the pistil and staminate flower parts mature at the same time and the structure and development of the flower facilitate transfer of pollen from the anthers to the stigma, sometimes even prior to opening of the flower. Although many crop plants are considered to be self-pollinating, such as cultivated tomato, rice, soybean, wheat, and barley, there are few that are completely self-pollinating, as some level of gene flow can generally be detected. In addition, there is variation for these traits within a species and its inter-fertile relatives. For example in pepper and tomato, the length of the pistil relative to the anther cone surrounding it can vary among varieties, with extrusion of the stigma beyond the anther cone facilitating gene flow. Furthermore, species such as safflower are considered to be self-pollinating, yet honeybees will travel up to 5 miles to collect its pollen (Chaney, 1985). If gene containment is necessary, effort is still required even for self-pollinating species as gene movement from crop to crop can be substantive, as has been shown for wheat, where 0.4% (0.0 to 4.2%) gene flow was detected in certified seed and 1.3% (0-11.3%) in farm-saved seed (Gaines et al., 2007; Willenborg and Van Acker, 2008). This discrepancy underscores the effectiveness of seed certification programs in maintaining seed purity. Nonetheless, self-pollinating plants generally require minimal mitigation strategies to contain pollen-mediated gene flow to acceptable levels for the seed industry with isolation distances less than 1320 feet (0.25 miles) resulting in gene flow less than 0.1%, the Foundation seed limit (see Appendices 4 and 5).

Outcrossing crops

Outcrossing crops can be wind-pollinated, as for many grass and chenopod species, or insect-pollinated. Gene flow in outcrossing crops has been re-visited with the introduction of transgenics for the reasons mentioned above, but also because new tools such as herbicide resistance allow for much better sampling, accuracy and statistical confidence to measure gene flow (Halsey et al., 2005; Van Deynze et al., 2005). For example, isolation distances for mitigating gene flow have been refined for corn (Halsey et al., 2005), cotton (Berkey et al., 2003; Van Deynze et al., 2011), canola (Rieger et al., 2002), alfalfa (Van Deynze et al., 2008) and sunflower (Reagon and Snow, 2006) based upon improved data using herbicide resistance as the marker (see Appendices 4 and 5). In general, pollen-mediated gene flow decreases exponentially with distance from the pollen source (i.e., it is inversely proportional to the distance) and is affected by the type of pollinator and pollinator activity (Van Deynze et al., 2005; Van Deynze et

al., 2008). Gene flow among related species has also been shown to be asymmetric; for example, when outcrossing occurred, Pima (*Gossypium barbadense*) cotton was preferentially pollinated by upland (*G. hirsutum*) cotton compared to the reverse (Van Deynze et al., 2011). Moreover, temporal isolation is an effective means to mitigate gene flow. For example, gene flow was reduced from 1% to 0.1% in maize by offsetting planting (flowering) by 7 days (Halsey et al., 2005).

Case studies

Alfalfa

Prior to the deregulation of alfalfa genetically engineered for resistance to glyphosate herbicide in 2005, the first perennial and obligate outcrossing transgenic crop, extensive research was done to study gene flow and trait persistence in the environment (Van Deynze et al., 2004). For example, gene flow using the primary pollinators (leafcutter bees and honeybees) in the main seed growing areas, establishment and removal of herbicide tolerant alfalfa, control of feral plants, management of herbicide-tolerant weeds and shifts in weed species and seed production and dormancy were studied and results were published (Teuber et al., 2007; Van Deynze et al., 2008; Van Deynze et al., 2004). In alfalfa, the number of honey bee visits decreases exponentially with distance from the hive (Hagler et al., 2011). Gene flow is directly correlated to the number of bees foraging (Teuber et al., 2011) and the type of pollinator. When alfalfa seed production fields are pollinated with leafcutter bees in the Northwest US, gene flow decreases below 0.5 % at 1000 feet with no gene flow detected at 2000 ft. In California, where honeybees are used as pollinators, gene flow decreases from 1.5% at 900 ft. to below 0.5% only at 2000 feet. It continues to decline exponentially to 0.03% at 15,840 ft. (3 miles) and not detected at 26,400 ft. (5 miles, Van Deynze et al., 2008). Although these studies indicate that gene flow can be maintained at very low levels with 0.5 miles (2,640 ft.) of isolation, the industry has voluntarily elected to use a 5-mile isolation zone for production of transgenic alfalfa seed using honeybees as pollinators. These preliminary studies were verified in field-scale experiments in 300 seed lots in eight western states where gene flow ranged from 0.0 to 0.2% when the industry Best Management Practices were used (National Alfalfa & Forage Alliance, 2008). Furthermore, areas such as the Imperial Valley in California (where other transgenic crops are grown) have elected not to grow transgenic alfalfa hay or seed in order to avoid potential disruptions in large non-GE/organic export markets. This type of self-regulation that responds to market demands independently of legal regulatory requirements is typical in the seed industry (see The Seed Industry Model above).

It is important to note that although alfalfa is the 4th largest crop (by area) in the U.S., only 1% of the crop is produced for seed, mainly in the western states. Gene flow from and to hay crops is an order of magnitude lower than among seed fields, as hay fields are usually cut prior to bloom and little viable seed is found in hay (Teuber et al., 2007; Van Deynze et al., 2008). For example, in experimental field tests in California, using honeybees as pollinators and simulating worst case scenarios where hay was allowed to grow to 20-50 % bloom, gene flow to adjacent seed fields at peak bloom at 165 ft. was <0.5% and 0.01% at 350 to 600 ft. In commercial fields, gene flow from hay to seed was at least 10 fold less than between seed fields (Teuber et al., 2007; Van Deynze et al., 2008). As hay fields are routinely cut prior to seed maturation (due to a reduction in hay quality with blooming), the chance of gene flow is reduced to non-detectable in

most cases (Putnam, 2006). The exposure or risk for gene flow in alfalfa for hay is therefore drastically reduced compared to alfalfa grown for seed.

Alfalfa has no sexually-compatible relatives in the U.S.; therefore, outcrossing is limited to neighboring fields and feral plants. Feral plants are common on roadsides and provide an opportunity to harbor and maintain transgenes in populations (Bagavathiannan and Van Acker, 2009; Bagavathiannan et al., 2011a). Although fecundity and seedling establishment were reduced in feral populations due to reduced pollination and allelopathic effects, feral alfalfa populations may have increased adaptation for survival traits such as overwintering (Bagavathiannan et al., 2010b). Mowing of feral alfalfa or spraying with herbicides was an effective method of reducing feral populations but will not necessarily eradicate them, given alfalfa's ability to maintain a seed bank (Bagavathiannan et al., 2011b; Bagavathiannan et al., 2010a). The relative size of feral populations (tens of plants) provides limited attraction and pollen source for pollinators relative to cultivated fields, resulting in a large dilution of gene flow from feral plants. However, unless completely controlled, feral plants provide an opportunity for persistence of transgenes in the environment. In areas of seed production, feral alfalfa populations are managed as prescribed by seed certification standards.

Cereals

Cereal crops, including corn, wheat, sorghum and rice, differ in their potential for gene flow (Mallory-Smith and Zapiola, 2008; Mallory-Smith and Sanchez Olguin, 2011, see Appendices 2,3 and 4). Although both sorghum and corn are outcrossing, corn has no cross-compatible species in the US, whereas sorghum can cross with other crop species including forage Sudan grass (*S. bicolor* ssp. *drummondii*) as well as the invasive weed species Johnson grass (*S. halpense*) and shattercane (*S. bicolor* ssp. *arundinaceum*). Current cultivars of sorghum have not been invasive, so breeding for increased biomass is not considered to be a risk. Adding traits that may give a natural selective advantage, such as tolerance to drought and salinity, may pose a risk if outcrossed to invasive weedy species. Similarly risk assessments for traits that may compromise control of weedy cross-compatible relatives should be considered, e.g. herbicide resistance. Risk management studies conducted in parallel with agronomic trials should not be overly burdensome to developers, growers, refiners, or regulators, but will require their collaboration to ensure that biofuel crops are produced sustainably with an acceptably low risk of invasiveness.

Biofuel Crops

Perennial and annual grasses are being evaluated for their potential to produce large amounts of cellulosic biomass to be converted to biofuels. The main traits being selected for are vigor, rapid establishment and growth, and production of digestible cellulosic biomass to be converted into alcohol biofuels. Except for the latter, these traits are also characteristic of many invasive grass species. The risk assessment of these selected crops and species therefore should be done in the target environments for growing them. Science-based risk assessment procedures are well established for potentially invasive plants based on matching climate and environmental models to each species' natural habitat and biology (Barney and DiTomaso, 2010b; Barney et al., 2011; DiTomaso et al., 2010). These include:

1. Determine the potentially invasive range using climate-matching analyses under various assumptions (e.g., drought tolerance) and scenarios (e.g., irrigation, climate change).

2. Evaluate environmental tolerance (e.g., soil moisture stress) of target biofuel crops.
3. Quantify invasiveness in susceptible habitats (e.g., riparian areas, woodlands, rangelands).
4. Perform propagule biology studies (e.g., seeds, rhizomes, stem fragments).
5. Assess hybridization potential with related native and non-native taxa.
6. Evaluate competitive interactions with desirable species within specific habitats.

Based on these studies, it is predicted that although switchgrass (*Panicum virgatum*) is marginally tolerant of low moisture conditions once established, it is unlikely to be invasive except perhaps in riparian environments where moisture is present throughout the year and competition with resident vegetation is low (Barney and DiTomaso, 2010a). Furthermore, it also is unlikely to be invasive in cultivated conditions because of its inability to compete with faster establishing crop plants and its susceptibility to tillage practices during the early years of growth. While seeds are the primary dispersal propagules, plants can also propagate by rhizomes under high moisture conditions. Switchgrass is a native species to the Midwestern U.S. and has coexisted with several congeneric species, yet has not been reported to cross with any other *Panicum* species, either native or introduced.

Another proposed biofuel species, giant miscanthus (*Miscanthus x giganteus*), is even less tolerant of water stress and only thrives in high moisture environments without competition. Unlike switchgrass, it is sterile and does not pose a risk via seed dispersal. While it does propagate via rhizomes fragments, it does not produce new shoots from older stem fragments, and has no sexually-compatible relatives in the United States. These characteristics greatly limit its potential to be invasive under cultivated conditions or in natural areas within Mediterranean environments. In contrast, giant reed (*Arundo donax*), also being considered for biofuels, is highly invasive in California and Texas and while it does not produce viable seed, it readily regenerates from each stem node and is easily distributed by plant fragments (Boose and Holt, 1999). No studies of within-species gene flow have been reported for the grasses mentioned above.

Trees

Transgenic trees for the traditional forest industry as well as the emerging renewable energy industry are being developed and tested in order to improve the sustainability and cost-effectiveness of producing woody biomass (Hinchee et al., 2010; Nehra et al., 2005). Though they have not been bred for the long time periods of many agricultural crops, some interspecies hybrid trees are highly domesticated, while others are hardly domesticated at all. However, most tree species have the capability to disperse pollen and seeds widely. Their size and mode of pollination, and their long-lived nature, often allow for gene flow among populations and sexually-compatible species over several kilometers. Pollen can travel several kilometers in forest trees, especially those which are wind-pollinated. For example, using paternity analysis Slavov et al. (2009) reported that approximately half of the pollen that fertilized seeds came from beyond 1 km in an area of dense cottonwoods in western Oregon, and approximately one-third came from beyond 10 km in an area of widely dispersed cottonwoods in eastern Oregon.

Combined with regulations, market restrictions for lumber and energy produced from transgenic trees, and the amenability of intensively grown species such as poplar, eucalyptus, and pine to

commercial vegetative propagation, there has been considerable interest in producing male-sterile or completely sterile trees. Such trees might not only enable sexual containment, but grow faster due to reduced investment in reproductive organs. Several promising approaches are underway for engineering complete sterility by RNA interference or directed mutagenesis against essential floral genes such as production of proteins with dominant negative amino acid substitutions or suppressor amino acid motifs (reviewed in Brunner et al., 2007) A new approach is to specifically mutate such genes essential for reproduction by directed mutagenesis approaches (e.g., zinc finger or TALEN nucleases), which appear to have high efficiency at gene targeting and mutation. Additionally, high levels of efficiency (in laboratory studies) for pollen-associated excision of transgenes have been previously reported (Moon et al., 2010) as an approach to floral sterility. Multiple-year field trials with ablation-based sterility systems -- where an anther-specific promoter drives the expression of a cell toxic gene such as barnase (Mariani et al., 1990) in its native or attenuated form have shown that complete or nearly complete pollen sterility can be achieved. Field data supporting this view have so far been obtained in poplar (Brunner et al., 2007), pine, and eucalyptus (Zhang et al., 2012). The ablation strategy is the most developed in trees; however, research is complicated by the need to evaluate performance in field experiments over multiple years under contained conditions. None of these systems are currently deregulated in trees in the US, though cold-tolerant and male-sterile eucalypts are currently under review for deregulation by USDA APHIS.

Gene Flow Mitigation Strategies

For most crops, the underlying biology and practical management of gene flow are well understood, with many examples of segregation to meet defined market thresholds, such as among field corn, sweet corn and popcorn. Gene flow mitigation strategies are utilized to the extent required to meet market requirements, particularly when there is a market premium for higher purity. This is exemplified by vibrant seed and identity-preserved specialty markets worldwide. In general, the costs of preventing gene flow and other admixtures in such markets have been in line with the premiums that segregated commodities have received (Kalaitzandonakes and Kaufman, 2006)

For new or developing cases (as mentioned above), risk assessment procedures are well established as are proven methods for segregation and gene flow mitigation. However, diverse international regulatory schemes for transgenes and markets with zero-tolerance thresholds, combined with the ability to detect transgenes at extremely low levels, have complicated trade as pragmatic product-based thresholds utilized in the past are no longer sufficient. Still, existing segregation systems may provide models on which to build.

Biological Gene Flow Mitigation Strategies

Biological gene flow mitigation strategies were first discovered in nature through self-incompatibility systems found in many species that prevent successful fertilization by the same or closely related plants. Such self-incompatibility systems have been utilized, particularly in Brassicas, to produce hybrid seeds (Nasrallah and Nasrallah, 1988). However, their complexity, due to the multiple alleles and loci involved, has limited their use to carefully controlled seed production fields. Natural fertility control systems based on male sterility have been discovered and utilized in many crops (corn, rice, onions, carrot, brassicas, cucurbits, etc.). These systems are used to control pollination between plants to produce hybrids. Recessive male-sterility genes

have been utilized, but these must be maintained by heterozygous sister lines and require roguing of fertile segregants in the field. Alternatively, cytoplasmic male sterility (CMS) systems, commonly due to non-functional mitochondrial genes that often are derived from crosses to distantly related species, have been widely adapted for hybrid seed production, as in maize, sunflower, Brassica, radishes, carrots and onions. Complementary naturally-occurring dominant nuclear restorer genes to overcome the CMS are introduced for effective crop production where fertility is required to allow production of fruits/seeds in farmers' fields, as in sunflowers, maize, and oilseed rape. Although effective, CMS can be unstable under high temperatures as in Brassicas (Niewhof, 1990). In maize, the widely used T-cytoplasm CMS source was associated with susceptibility to Southern leaf blight that resulted in epidemic failure of the maize crop in the 1970s (Weider et al., 2009). In reversible male-sterility systems such as CMS, restorer genes will segregate in subsequent generations, allowing for fertile progeny and thus gene flow in following generations. Gene flow can be limited during seed production of genetically engineered hybrids (e.g., sugar beets) by having the engineered gene only in the male-sterile female parent. An interspecific genetic incompatibility system is being studied to control gene flow in maize that creates male and female crossing barriers depending on the allelic makeup of specific genes, including *teosinte crossing barrier1 (tcb1)*, *gametophyte factor1 (gal)*, and *ga2* (Evans and Kermicle, 2001; Irish et al., 1994; Kermicle, 2006; Kermicle and Evans, 2010). Ploidy level can be used to mitigate gene flow via seed as in seedless watermelon, where a seedless triploid product is produced. On the other hand, it has been shown that herbicide resistance was introgressed from tetraploid *Brassica napus* to its diploid progenitor *B. rapa* (Warwick et al., 2008).

Engineered Gene Flow Mitigation Strategies

Kwit et al. (2011) reviewed gene mitigation strategies and classified them into pre-hybridization and post-hybridization strategies. Pre-hybridization strategies include genic and cytoplasmic male sterility, delayed flowering, transgene excision and cleistogamy (pollination without flower opening). Post-hybridization strategies included transgene mitigation and selective terminal lines (e.g., V-GURTs, see below). With our increasing knowledge of the genetic control of plant reproduction, many novel systems are being developed and evaluated for control of pollen (Stewart, 2007; Verma and Daniell, 2007), seed (Lee and Natesan, 2006) and even flower production (Liu et al., 2008) to address gene flow mitigation. For example, delayed flowering has been suggested as a method to mitigate gene flow by modifying or naturally selecting or inducing mutations in the *Flowering Locus C (FLC)* or *TFL1* gene, a repressor of flowering (Boss and et al., 2006; Kim and et al., 2007). The use of such systems would be limited to determinate flowering crops or forage and biomass crops where seed and fruit are not the harvested commodity.

Genic male sterility

Disrupting pollen development using genetic engineering has been suggested for containing transgene escape and introgression (Daniell, 2002; Feil et al., 2003). Multiple methods have been used to prevent pollen formation or decrease pollen fertility via genic or cytoplasmic male sterility. Many male-sterile plants have been genetically engineered using constructs that disrupt the tapetum, a layer of cells found within the pollen sac that is essential for pollen development (reviewed by Daniell, 2002). The first transgenic male-sterile plant was generated by genetic engineering of tobacco plants with the chimeric ribonuclease gene (Mariani et al., 1990). Most

genic male-sterile plants have been achieved by using tapetum-specific promoters to drive the expression of toxic bacterial genes (e.g., *Barnase* from *Bacillus amyloliquefaciens* and diphtheria toxin A), resulting in no pollen formation (Hird et al., 1993; Koltunow et al., 1990; Lee et al., 2003). Since then, several genetic engineering efforts have been demonstrated to develop other genic male-sterility approaches and applications in plants. These include using cytotoxic *barnase* gene expression in pollen or anthers of poplar (*Populus*) trees and *Kalanchoe blossfeldiana* (Garcia-Sogo et al., 2010; Wei et al., 2007). Transgenic pollen ablation has been demonstrated by expression of the diphtheria toxin gene under the control of the LAT52 pollen-specific and putative pectin esterase promoter in tobacco (Twell, 1995; Uk et al., 1998). A promising system being developed for pollen ablation is based on expression of an EcoRI restriction endonuclease driven by a pollen-specific promoter, which has shown 100% pollen ablation in initial studies (Moon and Stewart, 2011), including a test cross in which there was just one “escape” among 30,000-40,000 progeny screened per event for one-third of the transgenic events generated (Stewart et al., 2012)

CMS and Maternal Inheritance

As noted above, male-sterile plants can also be generated via CMS (Chase, 2006) and can be utilized for limiting transgene escape via pollen dispersal (Feil et al., 2003). One approach to inducing CMS blocks the production of functional pollen using mutations in the plant mitochondrial genome (Hanson and Bentolila, 2004). Genetically engineered CMS has been developed for biological transgene containment as well (Ruiz and Daniell, 2005). This was achieved by genetic engineering of the tobacco (*Nicotiana tabacum* L.) chloroplast genome with the *phaA* gene coding for β -ketothiolase, which is known to confer CMS (Ruiz and Daniell, 2005). The transplastomic lines were normal except for the male-sterile phenotype, lacking viable pollen. Male fertility in the engineered CMS lines could be restored by increasing photoperiod, which enhanced acetyl-CoA carboxylase activity and diverted acetyl CoA from β -ketothiolase, thereby reversing male sterility.

Chloroplast genomes are maternally inherited in most crops. Chloroplast transformation facilitates both transgene bio-containment and high levels of transgene expression, without the possibilities for gene silencing or position effects (Clarke and Daniell, 2011; Verma and Daniell, 2007). Therefore, genetic modification by insertion of transgenes into the chloroplast genome offers an attractive solution for controlling pollen-mediated gene flow among crop varieties and their wild relatives. Transgenes have been stably integrated and expressed via the tobacco chloroplast genome to confer important agronomic traits, including herbicide, insect, and disease resistance, drought and salt tolerance, CMS and phytoremediation capability. Chloroplast genomes of a number of crop species, including cotton, soybean, carrot, eggplant, sugar beet, cauliflower, cabbage, oilseed rape, poplar, potato, tomato, tobacco, lettuce and other crops, have been successfully transformed (Clarke and Daniell, 2011; Verma and Daniell, 2007). Chloroplast transformation in cereal crops was first reported in rice (Lee et al., 2006) and more recently in wheat (Cui et al., 2011). Maternal inheritance of genetically modified chloroplast genomes enables efficient containment of transgene movement via pollen or seeds and the absence of any reproductive structures when foreign proteins expressed in leaves are harvested facilitates their safe production in the field (Arlen et al., 2007).

Two recent studies confirmed efficient control of maternal inheritance of transgenes in transplastomic tobacco (Daniell, 2007). Ruf et al. (2007) evaluated paternal transmission via pollen of transplastomic plastid-specific antibiotic resistance and green fluorescence traits, enabling visual screening of progeny. The selection system identified only six paternal transmissions among 2.1 million seedlings screened (frequency of 2.86×10^{-6}), indicating that plastid transformation provides an effective tool to control gene flow from GE crops.

Transgene excision

Site-specific recombinases linked to pollen-specific promoters allow excision of transgenes from pollen, preventing expression of transgenes in progeny seeds and mitigating pollen-mediated gene flow. For example, transgenes were effectively excised from pollen in tobacco using Cre recombinase (Mlynárová et al., 2006). Other recombinases, including ParA and PhiC31, have been shown to excise transgenes in plants and have the potential to be used for pollen-specific transgene excision (Kempe et al., 2010; Thompson and et al., 2003). One novel resolvase (CinHI), a nuclease which is involved in DNA recombination, was adapted for transgene pollen excision in plants (Moon et al., 2011).

Conditional Seed Viability

It has become apparent that male-sterility systems, i.e., those that focus on pollen ablation or removal of transgenes from pollen, address only half of the gene flow mitigation equation. For example, transgenic canola (*Brassica napus*) has been shown to be distributed outside of expected cultivation areas (Aono et al., 2006; Pessel et al., 2001; Schafer et al., 2011). Distribution of canola by roadsides likely indicates unintended seed spillage and subsequent establishment of feral populations. Varietal gene use restriction technologies (V-GURTs) are the most developed systems focused on conditional seed viability (reviewed in Hills et al., 2007). Originally patented as a “technology protection system” by USDA scientists (Oliver et al., 1998), the system was criticized for its potential to prevent farmers from saving seed from their crops. This negative connotation persists, even though the same system could be used to mitigate transgene flow via seeds. V-GURTs utilize a conditionally expressed toxin or enzyme targeting the embryo late in seed development, which renders the mature seed non-viable. The seed crop commodity can therefore be produced, but the seeds will not be capable of germinating. An important feature is a chemically inducible on-switch for the system, without which commercial seed production would not be possible. Whereas the initial patent used the *Cre-Lox* recombinase system to remove blocking DNA to activate the synthesis of a ribosomal inhibitor protein, there are various routes to achieve seed non-viability. One of the earliest studies showing that such a system could be effective was one using a “recoverable block of function” (Kuvshinov et al., 2001). Here, germination-specific expression of barnase prevents germination, but this function can be blocked by barstar expression -- in this case, under the control of a heat-shock promoter.

Although genic and CMS male sterility and seed non-viability systems are considered effective gene flow mitigation strategies, without a reversible mechanism, these systems are limited to crops harvested for their vegetative parts such as forage or biomass crops. The lack of pollen could create negative impacts on pollen-feeding insect food chain (Mlynárová et al., 2006). A potential drawback of using CMS as a biological transgene containment tool is the potential for transmission of the transgene from the cytoplasm to the nucleus. Transmission of paternally-inherited plastids and mitochondria in crosses involving parents with an alien cytoplasm occurs

at low frequency (10^{-4} to 10^{-5}) in many plant species (Svab and Maliga, 2007). The loss of fertility in a CMS breeding plant population might therefore eventually be restored under natural conditions (Schnable and Wise, 1998). Furthermore, strategies that include cellular toxins may have potential toxicity to non-targeted organisms or cells.

A survey of all US permits and notifications (18,104 since 1985) indicates that 206 conferring altered fertility have been issued for field testing in maize, eucalyptus species, European plum, loblolly pine, poplar, rapeseed, sorghum, sweetgum and switchgrass with 77 different gene construct combinations, many listed as “confidential business information” (USDA, 2012). Of all the genetically engineered strategies described, only the barnase system has been commercialized and de-regulated successfully for inducing male sterility in oilseed rape in Canada and the US (Mariani et al., 1990) with a reversible system for fertility restoration. The barnase system has also been approved (but not commercialized) in the US in chicory and maize. A DNA α -amylase affecting male fertility is also approved in maize. While it is envisioned that appropriate gene expression and effective gene flow mitigation can be obtained using engineered strategies, a more pressing question remains one of politics and perception: are engineered male-sterility and seed non-viability systems acceptable to the public for gene-flow prevention purposes? With the development of non-food crops such as for bioenergy feedstocks or those synthesizing pharmaceutical proteins, it might be that the first commercial use and need for such technologies could be to limit transgene spread.

Practical Implications of Gene Flow Mitigation Strategies

There can be significant practical considerations and costs associated with implementing gene flow mitigation strategies associated with co-existence and identity-preserved products. Agriculture is inherently complex and includes numerous production systems that pursue multiple product standards to meet the demands of dozens of markets, even for a single crop. A primary convention in the seed industry, for example, is that the grower and value chain requiring a higher purity or identity standard, and therefore generally also commanding a higher market value, is responsible for meeting the required standard. The higher market value of the product compensates for the additional expense required to meet the standard (Kalaitzandonakes and Kaufman, 2006). In the seed industry, identity preservation is achieved by cooperation among neighbors and other growers of the same crop to synchronize isolation distances, planting dates, rotations, and other methods to minimize gene flow to meet genetic purity standards. In some situations, grower opportunity zones (GOZ) may be established in which the majority of growers in an area self-select to produce a certain commodity to capture specific higher value markets. For example, GOZs for production of sweet corn (vs. grain corn) seed have long been established in Idaho and are being established in the Imperial Valley of California for non-GE alfalfa seed.

As a practical consideration, the footprints of isolation zones associated with gene flow mitigation can have considerable impact. As the area of an isolation zone increases with the square of its radius ($\text{area} = \pi r^2$), a 5-mile isolation zone encompasses at least 78 square miles or 50,265 acres, compared to only 3.14 square miles or 2010 acres for a 1-mile zone (ignoring the size of the isolated field itself). The sizes of isolation zones, which are directly related to the degree of purity required in the final product, can therefore have large impacts on the crop choices of surrounding farmers or on the feasibility of producing a particular crop product in a

specific location. For this reason, seed production areas are often distinct from areas of concentrated production of the same commodity crop when large isolation zones are required. Isolation zones should therefore be adjusted according to science-based, crop-specific isolation distances and pragmatic purity thresholds should apply. Implementation of isolation zones must also be flexible and location-specific. For example, limiting certain crops or production systems on a county-wide basis, as was proposed (but not adopted) for herbicide-tolerant alfalfa, would have impacted crop choices of farmers on over 50% of alfalfa production acreage in California (D. Putnam, personal communication).

Organic, conventional and transgenic crops all have a place in U.S. agriculture, and practical gene flow mitigation strategies must be appropriately considered and deployed. Enactment of strict liability approaches that could displace cooperation and co-existence may be avoidable with voluntary identity preservation practices paired with biological strategies to limit gene flow. Practices that have long been used in seed and specialty crop production to maintain isolation and genetic purity provide models for how this can be achieved (see “The Seed Industry Model”).

Diverse Markets and Economic Considerations

Limiting the gene flow between neighboring crops has economic value in the production of planting seeds, of crops with special functional characteristics (e.g., waxy and high amylase corn), of organic crops with specific tolerances for transgenics and markets, and of non-transgenic crops that seek to avoid mandatory GE labeling or pursue GE-free voluntary labeling. Consequently, changes in farm operations for preventing gene flow as well as testing and remediation that are typically implemented in such production systems involve additional segregation costs that can be both direct and indirect (Kalaitzandonakes et al., 2001). Direct costs are payable costs and result from the re-engineering of operations, additional coordination and control (e.g., contracting costs, testing costs, third party certification fees, etc.), and liabilities from product failures (e.g., demurrage costs, costs of dispute resolution, etc.). Indirect segregation costs are non-payable and result from efficiency losses (e.g., underutilization of land due to use of buffer zones) and lost markets (Desquilbet and Bullock, 2002; Kalaitzandonakes et al., 2001).

The cost of segregation can vary significantly across commodities, regions, and over time, and a number of factors can influence their relative size. For instance, while controlling outcrossing may require expensive measures in the production of cross-pollinating crops as corn, it is a minor issue for self-pollinating crops such as soybeans. Similarly, testing costs might be significantly higher for non-transgenic corn than for soybean due to greater number of transgenic events that must be tested for in corn. The most significant driver of such costs, however, is the tolerance or threshold level. Low tolerances also mean additional testing and greater numbers of product failures (Desquilbet and Bullock, 2002; Kalaitzandonakes N.G. and Magnier, 2004). As tolerances diminish beyond certain levels, segregation costs increase exponentially (Kalaitzandonakes and Kaufman, 2006; Kalaitzandonakes N.G. and Magnier, 2004). Under zero or near zero tolerances, production and trade of segregated crops will tend to cease (Magnier et al., 2009). In addition, as all tests have some probability of false-positive results, the occurrence of false-positive detection in assays limits the practical minimum threshold that can be accurately monitored (Lamb and Booker, 2011).

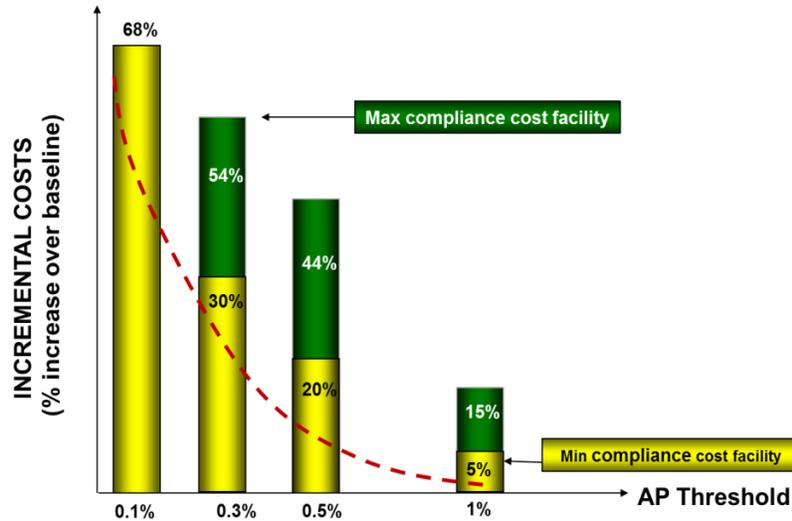


Figure 1. Incremental cost incurred over standard practices (baseline) in segregated seed corn production programs. The cost of compliance increases exponentially with decrease in thresholds (adapted from Kalaitzandonakes N.G. and Magnier, 2004).

Coexistence costs are also embedded in the expenses associated with the regulatory approvals of transgenic varieties (Kalaitzandonakes et al., 2007). Regulatory compliance costs are large and can discourage the development of transgenic crops/traits with limited market size (Bradford et al., 2006; Miller and Bradford, 2010). The costs of segregating transgenic materials during development and testing prior to deregulation can be substantial in their own right, as a zero threshold is mandated. These costs are incurred during field, greenhouse and transport of regulated materials for 5-10 years prior to commercialization of individual transgenic traits (currently on an event-by-event basis), regardless of the trait. Once a transgenic trait is approved to be grown, it is allowed to be combined with non-transgenic commodities and marketed unsegregated in the US and other countries where it is approved. When the same commodities are to be marketed in countries where certain transgenic events are not approved, they must be channeled or segregated prior to entering those markets, i.e., stewardship programs must be developed to track the product from farm to final destination (Sundstrom et al., 2003). Failures to meet set standards and tolerances in segregated programs can result in market losses and liabilities. Such liabilities may involve legal claims or proposed compensation funds and little is currently known about their potential economic implications.

Summary

The increasing complexity of U.S. and global agriculture requires coordination and application of practical co-existence systems. Gene flow is a natural two-way process that occurs among plants. Its impacts are specific to traits, crops, production systems and environments. **Gene flow mitigation strategies have been developed and are well-established in the seed industry and currently serve the agricultural industry well for domestic and international trade in cropping systems.** They serve as a model for developing crops. They are based on knowledge of the agricultural system, practical thresholds, coordination, and responsibility bestowed on the sector requiring higher standards, which usually have higher market value to compensate for

greater production costs. **Emerging production systems and products such as transgenic biofuel crops and trees may require novel gene mitigation strategies.** Current and emerging biological technologies show promise to minimize gene flow in agricultural production systems when needed. **Balanced risk assessments to evaluate both benefits and impacts of new traits on the environment and agriculture are required.** These will determine the appropriate gene flow mitigation strategy needed.

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Appendix – Meeting Agenda

The Science of Gene Flow in Agriculture and Its Role in Co-existence Washington, DC -- September 7-8th, 2011

SEPTEMBER 7th, 2011

	Topic	Speaker; Institution
8:30 AM	Welcome and meeting goals	Allen Van Deynze, UC Davis
8:40 AM	Opening remarks	Catherine Woteki, USDA Chief Scientist, Undersecretary for Research, Education and Economics
Impact of Gene Flow on Agriculture (Moderator: Allen Van Deynze)		
8:55 AM	Economic impact of gene flow	Nicholas Kalaitzandonakes, University of Missouri
9:20 AM	Maintaining seed purity in the seed trade industry	Ric Dunkle, American Seed Trade Association
9:45 AM	The implications of gene flow on organic farming	Mark Lipson, OSEC-USDA Organic and Sustainable Ag Policy
10:10 AM	BREAK	
10:30 AM	Importance of gene flow to germplasm conservation and development	Stephanie Greene, USDA/ARS, Prosser, WA
10:55 AM	The potential impact of gene flow mitigation on agriculture	Kent Bradford, UC Davis
11:20 AM	Panel discussion: Impact of Gene flow on Agriculture	
Gene Flow In the Environment (Moderator: Neal Stewart)		
11:45 AM	Outcrossing to wild relatives	Alison Snow, Ohio State
12:10 PM	LUNCH	
1:10 PM	Potential for persistence of genes in the environment	Joe DiTomaso, UC Davis
1:35 PM	Movement of genes in grasses	Carol Mallory-Smith, Oregon State
2:00 PM	Movement of honeybees in alfalfa	James Hagler, USDA/ARS, Maricopa, AZ
2:25 PM	Gene flow in alfalfa	Larry Teuber, UC Davis
2:50 PM	Challenges of organic alfalfa seed production	Ray Johnson, TopNotch Seed, Holtville, CA
3:15 PM	BREAK	
3:35 PM	Gene flow between feral and cultivated alfalfa populations	Rene Van Acker, University of Guelph
4:00 PM	Panel discussion: Gene flow in the environment	
Gene Flow Mitigation Strategies (Moderator: Kent Bradford)		
4:25 PM	Overview of male sterility strategies	Neal Stewart, University of Tennessee
4:50 PM	Non-transgenic cross incompatibility systems in maize	Matt Evans, Stanford University

5:15 PM Transgenic flower sterility strategies Zhongchi Liu, University of Maryland

6:00 PM **Social and discussions-Whitten Patio**

SEPTEMBER 8th, 2011

8:30 AM Workshop announcements Allen Van Deynze

Gene Flow Mitigation Strategies (continued)

8:35 AM Male sterility in hybrid systems Mark Albertsen, Pioneer Hi-Bred

9:00 AM Transgenic seed sterility strategies Mike Portereiko, Ceres, Inc.

9:25 AM Reversible male sterility using chloroplast transformation Henry Daniell, University of Central Florida

9:50 AM **BREAK**

10:15 AM Transgenic containment in trees Steve Strauss, Oregon State

10:40 AM Panel discussion: Gene flow mitigation strategies

11:05 AM General discussion-Impact, gene flow and mitigation strategies Allen Van Deynze

11:30 AM **Close**

Appendix 2. World's 25 most important food crops and their sexually compatible weed species¹. Adapted from Gealy et al. (2007)

Rank	Crop	Scientific Name	World Area Planted ² (M Ha)	World Yield ² (MT)	Related Weeds: Sexually Compatible with Crop ³	Rank Among World's Worst Weeds ⁴	Geographical Distribution
1	Wheat	<i>Triticum aestivum</i> <i>T. turgidum durum</i>	208	557	<i>T. aestivum</i>	>180	Nepal
					<i>Aegilops cylindrica</i>	>180	Turkey and U.S.
					<i>A. tauschii</i>	>180	Mediterranean: Iran
					<i>A. triuncialis</i>	>180	Mediterranean: Morocco and Turkey
					<i>A. ventricosa</i>	>180	Mediterranean: Morocco
2	Rice	<i>Oryza sativa</i> <i>O. glaberrima</i>	151	585	<i>O. sativa</i>	77-180	Worldwide: >50 countries
					* <i>O. glaberrima</i>	>180	W. Africa
					<i>O. barthii</i>	77-180	Subsaharan Africa: Nigeria
					<i>O. longistaminata</i>	>180	Subsaharan Africa
					<i>O. rufipogon</i>	77-180	Continental and insular Asia to New Guinea and north Australia, Latin America, Bangladesh
3	Maize	<i>Zea mays</i>	141	636	<i>O. punctata</i>	77-180	Nigeria and Swaziland
					* <i>Z. mays ssp. Mexicana</i>	>180	Mexico
4	Soybean	<i>Glycine max</i>	84	190	<i>G. soya</i>	>180	Northeast Asia: Korea, Taiwan, Japan northeast China; Russia (Siberia); Japan; Argentina
5	Barley	<i>Hordeum vulgare</i>	55	139	<i>H. spontaneum</i>	>180	East Mediterranean to Iran and west central Asia: Iran and Jordan
6	Sorghum	<i>Sorghum bicolor</i>	44	59	<i>S. bicolor</i>	>180	Africa and U.S.
					<i>S. alnum</i>	>180	Argentina, Australia, South Africa, and U.S.
					<i>S. halepense</i>	Top 18	Worldwide: 51 countries, native Southwest Asia and adjacent Africa
7	Millet	<i>Eleusine coracana</i> <i>Pennisetum glaucum</i>	35	29	<i>S. propinquum</i>	>180	Southeast Asia: Philippines
					* <i>E. coracana ssp. Africana</i>	>180	W. Africa
8	Cottonseed	<i>Gossypium hirsutum</i>	32	57	<i>P. sieberanum</i>	>180	W. Africa and north Namibia
					* <i>G. hirsutum, feral</i>	>180	Mesoamerica and Caribbean

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		<i>G. barbadense</i>			<i>G. tomentosum:</i> <i>Compatible?</i>	>180	U.S.
9	Beans, dry, green, and snap	<i>Phaseolus vulgaris</i>	28	26	<i>P. vulgaris: weed-crop-wild complex</i>	>180	Peru and Columbia
10	Groundnut (peanut)	<i>Arachis hypogaea</i>	26	37	<i>A. hypogaea</i>	>180	Taiwan
11	Rapeseed (canola)	<i>Brassica napus,</i> <i>B. rapa</i>	24	36	<i>B. napus</i>	>180	Europe, Argentina, Australia, Canada, U.S., 7 countries
					<i>B. juncea</i>	>180	Australia, Argentina, Canada, Fiji, Mexico, and U.S.
					<i>B. rapa (B campestris)</i>	77-180	Worldwide (temperate climate): >50 countries
					<i>Hirschfeldia incana (B. adpressa)</i>	>180	Europe, Australia, southern Africa, Argentina, and U.S.
					<i>Raphanus raphanistrum</i>	77-180	Worldwide (temperate climate): 65 countries
					<i>Sinapis arvensis (B. kaber)</i>	77-180	Worldwide (temperate climate): 52 countries
12	Sunflower seed	<i>Helianthus annuus</i>	21	26	<i>H. annuus</i>	>180	Mexico, South America, U.S., 11 countries
					<i>H. petiolaris</i>	>180	U.S.
13	Surgarcane	<i>Saccharum officinarum</i>	20	1350	<i>S. officinarum</i>	>180	Taiwan
					<i>S. spontaneum</i>	77-180	Asia, Africa, Middle East, Mesoamerica, 33 countries
14	Potato	<i>Solanum tuberosum</i>	19	311	None		
15	Cassava	<i>Manihot esculenta</i>	17	188	<i>M. esculenta</i> * <i>Manihot spp.:</i> all * <i>M. reptans</i>	>180	Southwest U.S. south to Argentina
16	Oats	<i>Avena sativa</i>	13	26	<i>A. fatua</i>	Top 18	Worldwide: 56 countries, native to Europe, North America, Middle East, and Central Asia
					<i>A. sterilis</i>	Top 18	Europe, North America, Middle East, and Central Asia, 18 countries
17	Oil palm fruit	<i>Elaeis guineensis</i>	11	139	None		

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18	Coffee	<i>Coffea arabica</i> <i>C. canephora</i>	11	7	None		
19	Coconut	<i>Cocos nucifera</i>	11	50	<i>C. nucifera</i> ; feral populations	>180	
20	Chickpea	<i>Cicer arietinum</i>	10	7	None		
21	Sweet potato	<i>Ipomoea batatas</i>	10	137	<i>I. trifida</i>	>180	Central and South America: Honduras and Mexico
22	Cowpea	<i>Vigna unguiculata</i>	9	4	<i>V. unguiculata</i>	>180	Niger and Nigeria (roadside weed)
23	Olive	<i>Olea europaea</i>	9	17	<i>O. europaea</i>	>180	Mediterranean basin
24	Rye	<i>Secale cereale</i>	8	16	<i>S. cereale</i> * <i>S. montanum</i>	>180 >180	Argentina, Finland, Iran, Turkey, and U.S. Mediterranean basin east through Turkey to Iraq, Iran
25	Grape	<i>Vitis vinifera</i>	7	62	* <i>Vitis</i> spp. <i>V. aestivalis</i> <i>V. candicans</i> <i>V. hastata</i> <i>V. rotundifolia</i> <i>V. rupestris</i> <i>V. tiliifolia</i> <i>v. trifolia</i> <i>V. vulpina</i>	>180 >180 >180 >180 >180 >180 >180 >180 >180	U.S. U.S. Malaysia U.S. U.S. Honduras India U.S.

¹ Adapted from Warwick and Stewart (2005). Although the information presented here focuses specifically on crops used for human consumption, many of the same major crops also are used heavily as sources of animal feed. Some crops traditionally considered animal feed (e.g., alfalfa) also are consumed as food in some instances.

² Area of production (million ha) and world yield (million metric tons) for 2003 from the FAOSTAT Web site, <http://faostat.fao.org/default.jsp>.

³ All species, except those preceded by an asterisk (*), are listed as a weed in Holm et al. (1979), or listed as a weed in Global Compendium of Weeds at Web site <http://www.hear.org/gcw/index.html>.

⁴ Holm Classification: "Top 18": ranked 1 to 18 of worst weeds by Holm et al. (1977); "19-76": ranked 19 to 76 by Holm et al. (1977); "77-180" : ranked 77 to 180 by Holm et al. (1997); ">180" indicates not listed among the 180 worst weeds or not listed as a weed.

Appendix 3. Recent (2005–2010) studies that provide molecular evidence of introgression from nontransgenic crops to their wild or weedy relatives (adapted from Kwit et al., 2011)

Crop	Relative	Molecular marker	Refs.
Cichorium intybus	C. intybus	AFLP	(Sorensen et al., 2007)
Glycine max	Glycine soja	SSR	(Kuroda et al., 2006)
Helianthus annuus var. macrocarpus	Helianthus petiolaris	RAPD	(Gutierrez et al., 2010)
Medicago sativa	M. sativa	AFLP, SSR	(Greene et al., 2008)
Oryza sativa	Oryza rufipogon	SSR	(Song et al., 2006)
Pennisetum glaucum	P. glaucum	SSR	(Lewis, 2010)
Phaseolus vulgaris	Ph. vulgaris	AFLP	(Papa et al., 2005)
Raphanus sativus	Raphanus raphanistrum	Allozyme	(Snow et al., 2010)
Sorghum bicolor	Sorghum halepense	RFLP	(Morrell et al., 2005)
Triticum aestivum	Aegilops peregrine	Fragment of noncoding locus	(Weissmann et al., 2005)
Vigna unguiculata	V. unguiculata ssp. unguiculata var. spontanea	RFLP	(Feleke et al., 2006)
Vitis vinifera	V. vinifera ssp. silvestris	SSR	(Di Vecchi-Staraz et al., 2009)
Zea mays	Z. mays	SSR	(Bitocchi et al., 2009)

Abbreviations: RAPD, randomly amplified polymorphism; RFLP, restriction fragment length polymorphism; SSR, simple sequence repeat.

Appendix 4. Summary of result of field trials measuring pollen dispersal distance from transgenic plants (adapted from Chandler and Dunwell, 2008)

Crop	Observation	Citation
<i>Brassica napus</i>	Showed gene flow to no more than 3 km from 25–100 ha fields of non-GM HT crops.	Rieger <i>et al.</i> (2002)
	Glyphosate-resistant plant detected 500 m from a 0.5 ha transgenic plant plot.	Hall <i>et al.</i> (2000)
	Pollen-mediated gene flow to up to 800 m and persistence of seed volunteers, combining to lead to survival of double herbicide-resistant varieties.	Beckie <i>et al.</i> (2003)
	Measured pollen flow from individual plants. 50% of pollination occurred in less than 3 m.	Lavigne <i>et al.</i> (1998)
	Showed in a 100-square-kilometer survey that pollination can occur at distances of over 1 km.	Devaux <i>et al.</i> (2005)
Tall fescue	From a central transgenic plot surrounded by recipient plants, no transgene detected beyond 200 m.	Wang <i>et al.</i> (2004b)
Barley, wheat	No gene flow detected at distances greater than 12 m from small plots.	Gatford <i>et al.</i> (2006)
Sunflower	Measured sunflower gene flow to wild sunflower, in Argentina. High levels of hybridization at a few meters and detectable at up to 500 m.	Ureta <i>et al.</i> (2007)
Chinese cabbage	No evidence for gene flow in Chinese cabbage grown adjacent to transgenic Chinese cabbage. Compatibility proven by hand-pollination.	Lim <i>et al.</i> (2007)
Tomato	No evidence for any hybridization, even in adjacent plots.	Llardi and Barba (2001)
Bentgrass (<i>Agrostis stolonifera</i> L.)	Gene flow detected primarily at under 2 km and in sentinel plants up to 20 km away from transgenic source. Hybrids identified in recipient plots up to 3.8 km down-wind from transgenic HT control area.	Watrud <i>et al.</i> (2004) Reichmann <i>et al.</i> (2006)
Sugar beet	Up to nearly 300 m using bait plants.	Darmency <i>et al.</i> (2007)
Maize	Gene flow at detected at 200 m, using bait plants.	Saeglitz <i>et al.</i> (2000)
	Evaluated gene flow using ha size plots. Out-crossing occurred at 0.03–0.1% at a distance of 100 m.	Goggi <i>et al.</i> (2007)
Soybean	0.5% transmission of a HT gene at a distance of 1 m and 0% at 10 m.	Abud <i>et al.</i> (2007)
	No gene flow at distances over a few meters in transgenic soybean, most probably due to insect pollinators.	Yoshimura, (2006b)
Rice	Negligible transgene frequencies at less than 10 m.	Rong <i>et al.</i> (2007)
	Relatively little pollen flow beyond 10 m.	Messeguer <i>et al.</i> (2001, 2004)
	Measured gene flow in cultivation size plots of rice. Though majority of hybridization at under a few meters hybridization was detected at up to 150 m.	Jia <i>et al.</i> (2007)
Common bean (<i>Phaseolus vulgaris</i>)	Gene flow measured by flower color was virtually zero beyond a few meters (study used non-GM crops).	Ferreira <i>et al.</i> (2007)

Appendix 5. Minimum recommended seed isolation standards (in feet) for foundation, registered and certified generations (adapted from Sundstrom et al., 2003).

Crop	Foundation	Registered	Certified	Other considerations (see AOSCA standards)
Self pollinated				
Bean				
field and garden ¹	0	0	0	
lima	30	30	30	100 ft for Fordhook
cowpea ¹	0	0	0	
Bermudagrass	900	---	165	10% percent rule ²
Cotton				
same type ^{1,2}	660	660	20	20 buffer rows
different type ²	1320	1320	660	20 buffer rows
Peas (field) ¹	0	0	0	
Peanut ¹	0	0	0	
Pepper ³	200	100	30	
Rice ^{2,4}	10	10	10	Planting direction
Small grains				
barley, oats, triticale and wheat ¹	0	0	0	Hybrid barley
buckwheat and rye	660	660	660	Diploid and tetraploid rye
Soybeans ¹	0	0	0	
Tobacco				
self-pollinated	150	150	150	Varieties of same type
hybrid	---	---	150	Male sterile and fertile varieties
Tomato ³				
self	200	100	30	
hybrid	---	---	0	
Wind pollinated				
Corn				
inbred lines ⁵	660	---	---	varieties of same color and texture; dent corn
foundation ⁵				
single cross	660	---	---	varieties of same color and texture; dent corn
backcross	660	---	---	varieties of same color and texture; dent corn
hybrid	---	---	660	varieties of same color and texture; dent corn
open pollinated	---	---	660	varieties of same color and texture; dent corn
sweet	---	---	660	field size
Grasses				
cross pollinated ⁶	900	300	165	diploids and tetraploids; field size
apomictic/self fertile	60	30	15	diploids and tetraploids; field size
Millet				
cross pollinated ⁷	1320	1320	660	
self pollinated ¹	0	0	0	
Sorghum ²	990	990	660	pollinator parent; dissimilar types

hybrid seedstock	990	---	---	
hybrid	---	---	660	bloom time; pollinator parent; contaminating source

Crop	Foundation	Registered	Certified	Other considerations (see AOSCA standards)
Insect pollinated				
Alfalfa ^{2,8}	900		---	165 10% rule ²
hybrid ^{8,9}	1320		---	165 varietal adaptation region
Canola				
self pollinated ¹⁰	660		---	330
cross pollinated	1320		---	330
Clover (red and white) ²	900			165 diploids and tetraploids; field size
Okra	1320		1320	825
Onion	5280		2640	1320
Safflower	1320		1320	1320
Sunflower ^{2,11}				
open pollinated	7920			7920 oil and non-oil types; volunteers and wilds
hybrid	---		---	6600 oil and non-oil types; volunteers and wilds
restorer or maintainer lines	6600		---	---
male sterile	13200		---	---
Watermelon ^{2,12}	10560		5280	2640 citron ²

¹Adequate distance to prevent mechanical mixture is necessary.

²See California Crop Improvement Association 2012. ccia.ucdavis.edu

³Distance may be reduced by half if different generations of same variety are adjacent.

⁴Distance between fields of the same variety is 10 ft. if ground drilled, 50 ft. if ground broadcast and 100 ft. if aerial seeded.

⁵No isolation required for production of hand-pollinated seed.

⁶Isolation between classes of same variety may be reduced to 25% of distance otherwise required.

⁷Isolation between millets of different genus is 6 feet.

⁸Distances between different generations of same variety may be reduced to 10 ft.

⁹Parent lines in a crossing block, or seed and pollen lines in a hybrid production field must be separated by 6 feet or more.

¹⁰Required isolation between generations of the same variety is 10 ft.

¹¹Doesn't apply to *Helianthus similes*, *H. ludens* or *H. agrestis*.

¹²Minimum distance may be reduced by 50% if field is adequately protected by natural or artificial barriers.

Appendix 6. Summary of current technologies currently under development specifically to mitigate gene flow.

System	Crop	Regulatory status*	Reference
Control of pollen			
Chloroplast transformation	Tobacco	Experimental	(Daniell, 2007; Ruf et al., 2007)
Genic male sterility			
Barnase	Oilseed rape	Deregulated/commercialized	(Mariani et al., 1990)
Barnase	Chicory, maize	Deregulated	(Mariani et al., 1990)
Barnase	<i>Populus</i>	Regulated field trials	(Wei et al., 2007)
Barnase	<i>Kalanchoe blossfeldiana</i>	Experimental	(Garcia-Sogo et al., 2010)
Barnase	<i>Pinus taeda</i> , Eucalyptus	Regulated field Trials	(Zhang et al., 2012)
Diphtheria toxin gene	Tobacco	Experimental	(Twell, 1995)(Twell, 1995)
Transgene excision			
Cre recombinase	Tobacco	Experimental	(Mlynarova et al., 2006)
Restriction endonuclease	Tobacco	Experimental	(Stewart et al., 2012)
PhiC31 recombinase	Wheat	Experimental	(Kempe et al., 2010)
ParA recombinase		Experimental	(Thompson and et al., 2003)
CinHI resolvase	Tobacco	Experimental	(Moon et al., 2011)
Conditional seed sterility			
Cre-Lox	Tobacco, Cotton	Regulated field trials	(Oliver et al., 1998)
DNA adeninemethylase	maize	Deregulated	(USDA Biotechnology Regulatory Service, 2012)

*Experimental refers to in lab or greenhouse only.