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Review
Gene flow from glyphosate-resistant crops

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Abstract: Gene flow from transgenic glyphosate-resistant crops can result in the adventitious presence of the transgene, which may negatively impact markets. Gene flow can also produce glyphosate-resistant plants that may interfere with weed management systems. The objective of this article is to review the gene flow literature as it pertains to glyphosate-resistant crops. Gene flow is a natural phenomenon not unique to transgenic crops and can occur via pollen, seed and, in some cases, vegetative propagules. Gene flow via pollen can occur in all crops, even those that are considered to be self-pollinated, because all have low levels of outcrossing. Gene flow via seed or vegetative propagules occurs when they are moved naturally or by humans during crop production and commercialization. There are many factors that influence gene flow; therefore, it is difficult to prevent or predict. Gene flow via pollen and seed from glyphosate-resistant canola and creeping bentgrass fields has been documented. The adventitious presence of the transgene responsible for glyphosate resistance has been found in commercial seed lots of canola, corn and soybeans. In general, the glyphosate-resistant trait is not considered to provide an ecological advantage. However, regulators should consider the examples of gene flow from glyphosate-resistant crops when formulating rules for the release of crops with traits that could negatively impact the environment or human health.

Keywords: glyphosate resistance; gene flow; environmental impact

1 INTRODUCTION

Gene flow, defined as the change in gene frequency in a population due to movement of gametes, individuals or groups of individuals from one place to another,[1] has been raised consistently and repeatedly as a concern related to the introduction of genetically engineered (GE) crops.[2–4] Gene flow is a natural phenomenon that is not unique to GE crops. The concerns raised relative to gene flow from GE glyphosate-resistant (GR) (Roundup Ready®) crops include: the emergence of volunteer crops that are more difficult or more expensive to control, the transfer of the transgene to wild or weedy relatives and transfer of the transgene to conventional and organic crops. Gene flow to non-GE cultivars also may cause marketing issues because of the adventitious presence of a transgene. Gene flow can occur via pollen and seed (Fig. 1) and, for some species, may also occur via vegetative propagules. More emphasis has been placed on the potential for pollen to move transgenes. Although pollen is an important means of gene flow, the intentional movement of seed during commerce may be of greater importance for the long-distance dispersal of transgenes.[5] Gene flow via vegetative propagules has rarely been addressed, but it could be an important avenue for transgene movement.

The authors recognize that there are concerns in addition to gene flow from GR crops, such as the evolution of glyphosate-resistant weeds, or species shifts to more tolerant weeds because of the increased selection pressure from repeated glyphosate applications. Glyphosate-resistant volunteers also are an issue, especially in cropping systems with multiple GR crops. Because glyphosate is the herbicide most often used in no-till and minimum-till systems, GR volunteer crop plants and glyphosate-resistant or tolerant weeds will jeopardize the sustainability of those systems. Although the main focus of this article is gene flow as it pertains to GR crops in the USA, some references to management issues are included. The review will address crops that are commercially available or are in the process of deregulation in the USA.

1.1 Status of GR crops

Genetically engineered GR crops have been sold in the USA since 1994. The glyphosate resistance trait allows glyphosate, a non-selective herbicide, to be used on crops that would otherwise be killed by the herbicide. Glyphosate-resistant crops are resistant because they have a transgene that produces a herbicide-insensitive target-site enzyme, EPSPS (5-enol-pyruvylshikimate-3-phosphate synthase). The gene (*CP4 EPSPS*) was originally isolated from *Agrobacterium* sp. strain CP4.[6] Canola (*Brassica napus* L.) contains a glyphosate oxidoreductase (*GOX*) gene (*goxv247*) from *Ochrobactrum anthropi* strain LBAA in addition to the *CP4 EPSPS* gene.[7] The

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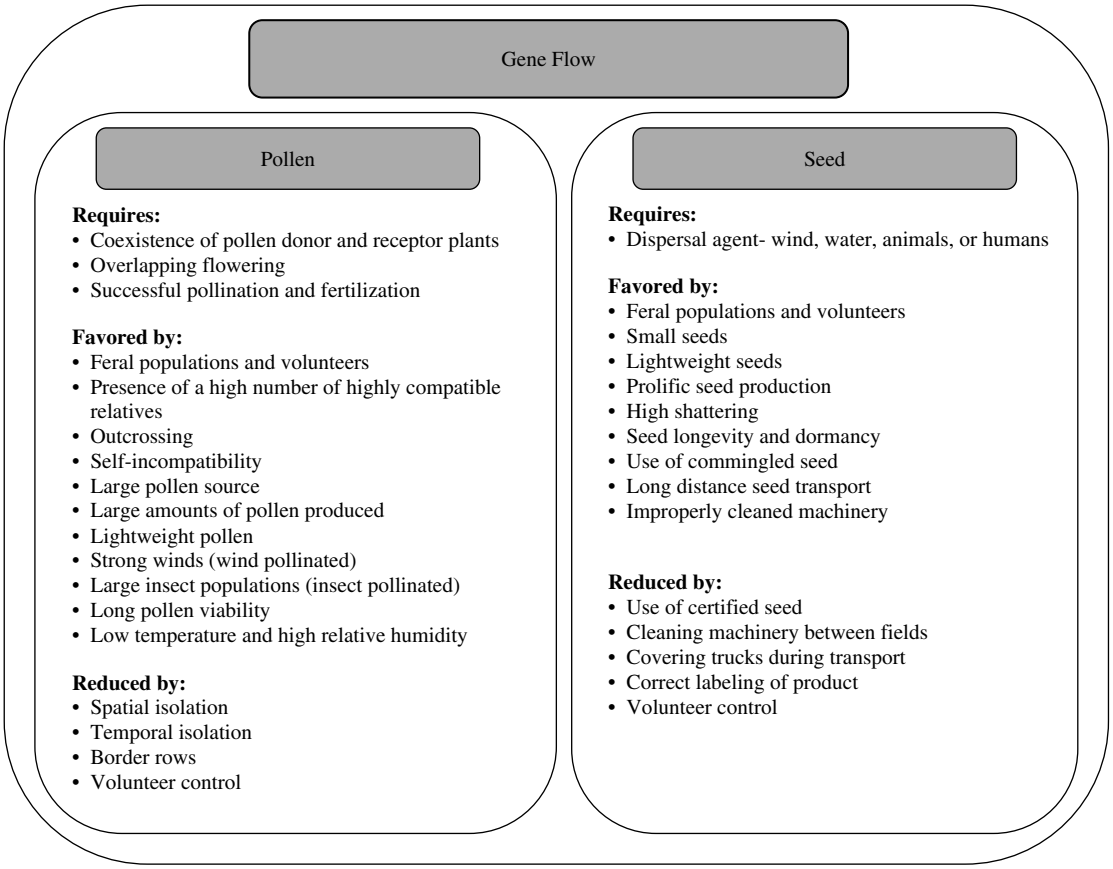

Figure 1. Gene flow via pollen vs seed. Comparison of the requirements and factors affecting gene flow via pollen and seed.

GOX enzyme metabolizes glyphosate to glyoxlate and aminomethylphosphonic acid.

Glyphosate-resistant crops approved for sale in the USA include canola, corn (*Zea mays* L.), cotton (*Gossypium hirsutum* L. and *G. barbadense* L.), soybean (*Glycine max* (L.) Merr.) and sugarbeet (*Beta vulgaris* L.). Alfalfa (*Medicago sativa* L.) was deregulated in 2005 and planted commercially in 2006. However, in March 2007, a court ruling returned its status to a regulated article.[8] Deregulation of GR creeping bentgrass (*Agrostis stolonifera* L.) has been requested but not yet approved.[9] Glyphosate-resistant wheat (*Triticum aestivum* L.) was under development; however, Monsanto withdrew its request for deregulation in 2004.[6]

1.2 Factors affecting gene flow

Gene flow is influenced by the biology of the species, the environment and production practices (Fig. 1). The biology of the GR crops varies (Table 1). Perennial crops, such as alfalfa and creeping bentgrass, differ from annual crops in that they persist in the field for more than one growing season and may reproduce by means of vegetative propagation as well as seed. These two characteristics allow them to contribute genes for gene flow for a longer period of time than annual crops. Outcrossing crops, both wind and insect pollinated, have a higher potential for gene flow via pollen than self-pollinated crops. However, gene flow via seed and vegetative propagules is independent of pollination type. The smaller the seed, the greater the risk for gene flow through natural dispersal mechanisms or human actions. Alfalfa, corn and soybean do not have wild or weedy compatible relatives in the USA, in contrast to canola, cotton, creeping bentgrass and sugarbeet. In addition, feral populations of alfalfa, canola and creeping bentgrass can be a continual source of the glyphosate resistance gene. The GR crops also have different production practices. Some crops such as canola, corn and soybean are mainly processed, while others, such as creeping bentgrass and alfalfa, are produced initially for their seeds which are then planted for turf or forage.

When gene flow from a GE crop occurs, via pollen, seed or vegetative propagules, it results in the adventitious presence of the transgene. Adventitious presence refers to low levels of unintended material in seed, grain or feed and food products (USDA (http://w3.usda.gov/agencies/biotech/ac21/reports/tlpaperv37final.pdf)). In the USA, minimum purity standards and allowable levels of seed of other varieties for each crop are set by the Federal Seed Act (http://www.ams.usda.gov/lsg/seed/seed_pub.htm#Regulations). Allowable levels of seed of other varieties differ by crop and whether the seed is foundation, registered or certified. For example, for cross-pollinated grasses, the allowable levels are 0.1, 1.0 and 2.0% for each seed class respectively; for alfalfa the levels are 0.1, 0.25 and 1.0% respectively. Often more stringent standards are set by industry and market requirements than those set by the Federal Seed Act. According

Table 1. Comparative biology of glyphosate-resistant crops

Common name	Species	Pollination type	Pollen vector	Compatible relatives in USA	Feral populations	Life cycle	Seed weight[a] (seeds g^{-1})
Alfalfa	*Medicago sativa*	Outcrossing	Insect	No	Yes	Perennial	500
Canola	*Brassica napus*	Outcrossing	Insect and wind	Yes	Yes	Annual	300
Corn	*Zea mays*	Outcrossing	Wind	No	No	Annual	3
Cotton	*Gossypium* spp.	Mostly selfing	Insects	Yes[b]	No	Perennial[c]	8
Creeping bentgrass	*Agrostis stolonifera*	Outcrossing	Wind	Yes	Yes	Perennial	13 500
Soybean	*Glycine max*	Selfing	None	No	No	Annual	10
Sugarbeet	*Beta vulgaris*	Outcrossing	Wind	Yes[b]	No	Biennial[c]	55

[a] Reference: Rules for testing seeds, Association of Official Seed Analysts, 2002.
[b] Limited distribution.
[c] Managed as annuals.

to the American Seed Trade Association, once a GE crop is deregulated, its seed is equivalent to that of a conventional crop. The adventitious presence of a regulated transgene is illegal.

The mixture of GE and non-GE seed can be described as commingling or admixture. Commingling can occur at planting, if seeds of a GR cultivar are mixed with seed of a non-GR cultivar. It also can occur if volunteer crop plants (i.e. plants that emerge from seed from a previous crop) produce seed that is harvested with the crop, or during post-harvest operations such as cleaning, conditioning, transport or storage. The number of processing steps that occur after harvest depends on the crop and its final use. Commingling in forage and straw also can occur.

1.2.1 Gene flow via seed

Seeds are moved either by natural dispersal mechanisms, such as water, wind or animals, or by human actions. In general, natural seed dispersal occurs at relatively short distances, in the order of meters, from production fields;[10] but, by being more persistent than pollen, seeds can be moved further distances.[5] Seeds of some GR crop species, such as creeping bentgrass, are small and thus more likely to be moved by natural dispersal agents than others. However, seed movement is difficult to prevent or predict. The intentional movement of seeds by humans during commerce results in an essentially limitless dispersal capability. The seed handling system is 'leaky', and seed loss can occur at any point from planting to final sale. The more steps that occur during production and post-harvest operations, the more opportunities there are for gene flow (Fig. 2).

Seed characteristics that influence gene flow are size, longevity, dormancy and seed shattering which occurs before or during harvest (Fig. 1). Some crops such as creeping bentgrass and canola are prone to shattering and will contribute large numbers of seed to the seedbank. Dormancy allows dispersal in time by maintaining genes year to year in the soil seedbank. The seedbank includes many generations of seed, thereby conserving genetic material. Some GR crops such as corn and soybean will not produce a persistent seedbank, while others such as canola, alfalfa and creeping bentgrass will produce persistent seedbanks that become a reservoir for the transgene.

1.2.2 Gene flow via pollen

The distance at which gene flow via pollen can occur is variable. It is impossible to predict the furthest distance that viable pollen can move. In general, gene flow via pollen will occur at relatively short distances because pollen is viable for a short time, generally only hours or days. Pollen is subject to desiccation, so viability is reduced by high temperatures or low relative humidity. Gene flow via pollen will increase with a wind-pollinated, outcrossing, self-incompatible species such as creeping bentgrass. Gene flow via pollen also will increase when there are: highly compatible relatives, many species of compatible relatives, synchronous flowering, a large pollen source and strong winds (Fig. 1).[10–14]

1.2.3 Gene flow via vegetative propagules

Gene flow via vegetative propagules of GE crops has not been studied in depth. Vegetative propagules, such as stolons, rhizomes, roots, crowns and bulbs, allow single plants to reproduce in isolation and become a source of the transgene. Short-distance movement can occur between fields via natural means or on equipment moved between fields. Long-distance movement would not be expected except with human intervention or possibly via waterways. However, there are examples of long-distance movement of propagules for planting. For example, Carrier[15] reported that viable creeping bentgrass stolons could be shipped anywhere in the USA if they were placed in soil, and reported receiving viable stolons shipped from Sweden to Washington State.

2 GR CROPS AND GENE FLOW

2.1 Soybean (*Glycine max*)

In 1994, GR soybean was the first GR crop to be deregulated in the USA. By 2006, 95% of the 30.5 million ha planted to soybean in the USA were GR [USDA-NASS (http://www.nass.usda.gov/QuickStats/index2.

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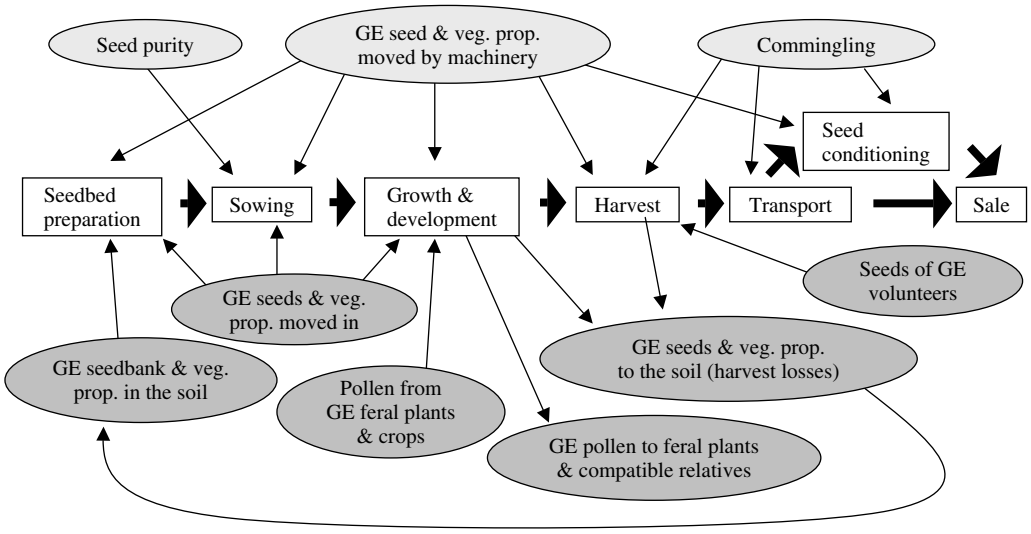

Figure 2. Steps during production and processing practices where gene flow can occur (GE = genetically engineered; veg. prop. = vegetative propagules). Adapted from Reference 34.

jsp)]. Therefore, transgene flow is an issue mainly if a grower wishes to produce conventional or organic soybean.

Soybean is an annual, highly self-fertile, self-pollinating species.[16] Pollination occurs either in the bud stage or before the flowers completely open. Soybean pollen is too heavy for wind transport;[17] however, pollination by honey bees (*Apis mellifera* L.) has been shown to increase the yield of some cultivars.[18] Gene flow via pollen generally has not been raised as an issue in soybean because, in addition to the low level of outcrossing, soybean is not found outside cultivation[19] and has no compatible relatives in the USA. Therefore, isolation distance for the production of foundation soybean seed is only the distance required to prevent mechanical mixing.[20] Nevertheless, it is apparent that gene flow via pollen can occur.[18] Cross-pollination in adjacent rows of soybeans ranged from 0.03% to 0.44% over 3 years in Arkansas.[16] In the same study, outcrossing was 0.01% at 10 m from the pollen source in one year, and 0.004% at 14 m in another year.

Soybean does not produce a persistent seedbank because the seeds lose viability quickly and have no dormancy.[21] The lack of dormancy allows soybeans to germinate and become volunteers if temperature and moisture are adequate. Volunteers can occur in subsequent crops, so crop rotation has been used to maintain genetic purity in seed production, both in GE and non-GE cultivars. Volunteer GR soybeans have been reported in cotton.[22] Commingling of volunteer GR soybean seed in cotton during harvest should not be an issue because of the difference in growth and production practices of the two crops.

To determine if non-GE seed stocks in the USA contain transgenes, a study was conducted by the Union of Concerned Scientists (UCS) to test for the adventitious presence of transgenes in canola, corn and soybean seed stocks.[23] Seeds from six non-GE seed varieties were tested by two independent laboratories for the presence of the *CP4 EPSPS* transgene as well as other GE traits. Although the UCS considered this a small pilot study and did not make any conclusions about the absolute levels of contamination, the results do certainly point out the difficulty in preventing transgene movement with seed stock.

The UCS study documented the presence of the *CP4 EPSPS* transgene, at levels of 0.05 and 1%, in 50% samples of the six conventional soybean cultivars.[23] Although it was not possible to determine whether gene flow via pollen or seed was responsible for the adventitious presence, commingling seems more likely to be the cause, based on the low level of outcrossing in soybean, the millions of soybean hectares harvested and the million of tons of soybean produced and handled in the USA.

2.2 Cotton (*Gossypium* spp.)

Glyphosate-resistant cotton was deregulated in the USA in 1995. Glyphosate-resistant upland (*Gossypium hirsutum*) and Pima (*G. barbadense*) cotton are grown in the USA. Most of the 6.2 million ha of cotton grown in the USA in 2006 were planted to upland cotton [USDA-NASS (http://www.nass.usda.gov/QuickStats/index2.jsp)]. Both species are managed as annuals that can be either self-pollinated or cross-pollinated by insects, most often bumblebees (*Bombus* spp.). Cotton pollen is sticky, and movement by wind is negligible. Reported outcrossing rates vary widely and depend on the number of pollinators present in a field, and possibly on the variety.[24,25] Required isolation for foundation seed is either a natural barrier or crop boundary, or 30 m if there is an easily observed morphological characteristic in the contaminating pollen source field. Isolation distance between upland and Pima cotton is 400 m for foundation and registered seed, and 200 m for certified seed.[20]

Simpson and Duncan[25] reported 47% outcrossing when averaged over sampling points, but outcrossing decreased as distance increased from the pollen source, and was <3% at 15 m. They also reported that pollen distribution depends on the foraging habits of the pollinator, not on its flight range. Meredith and Bridge[26] reported 0.0–5.9%, with an average 2.0%, outcrossing across 11 locations in Mississippi, and concluded that, in that area, cotton was essentially a self-pollinated species. Nevertheless, the range of outcrossing rates and the potential for insect pollination provide opportunity for at least some gene flow via pollen. Outcrossing rates since the introduction of GE insect-resistant (BT) cotton, which might require fewer insecticide applications, have not been reported. It would seem possible that the outcrossing rates might become significant if insecticide applications decrease and there is an increase in pollinator populations.

Although there are some wild populations of *G. hirsutum* in the USA, they are limited to Southern Florida.[27] A wild relative, *G. thurberi* Tod., occurs in Arizona, but it is a diploid species and is not compatible with cultivated cottons which are tetraploids.[28] Therefore, *CP4 EPSPS* gene flow via pollen to compatible relatives is not considered to be a significant issue in the USA.

Cotton seeds are not dormant and do not persist in the environment.[28] Volunteer cotton plants do occasionally occur in subsequent crops, but they generally will not survive winter temperatures.[28,29] Cotton seeds are relatively large, 8 seeds g^{-1},[30] and the greatest potential for commingling of cotton seed occurs during ginning if more than one variety is processed at a facility.[24]

2.3 Corn (*Zea mays*; maize)

Corn is the leading grain crop in the USA, with 32.1 million ha planted in 2006 [USDA-NASS (http://www.nass.usda.gov/QuickStats/index2.jsp)]. Glyphosate-resistant corn was deregulated in the USA in 1997, and, by 2005, 50% of the total USA corn crop was planted to herbicide-resistant hybrids, with GR corn accounting for the majority of the plantings.[31]

Corn is an annual, monoecious, outcrossing (5% self-pollinated), primarily wind-pollinated crop that produces abundant pollen.[32,33] As the cobs are protected by the husk, corn does not shatter and rarely sheds seeds, and therefore has a low potential for scattering seeds. However, corn grains or cobs left on the field after harvest can result in volunteer plants the following year.[34] Volunteer corn can compete with soybeans and cause yield loss.[35] Volunteer GR corn in GR soybeans requires an additional herbicide because glyphosate is no longer effective for volunteer corn control. Because corn seed has no dormancy, the potential to contribute to the seedbank is low.[34,36] Corn has several wild relatives in the genus *Zea*, commonly known as teosintes, with which it can cross.[37] Teosintes have limited distribution and have not spread beyond their natural range in Mexico and Central America,[37] and therefore there is no risk of gene flow from corn to wild relatives in the USA. Because corn does not persist outside cultivation, and feral corn populations are not common,[36,38] the main dispersal routes for the *CP4 EPSPS* gene in corn are via pollen between neighboring fields and by seed commingling.

Corn pollen is one of the largest and heaviest among the grass species, measuring approximately 100 µm and weighing around 0.25 µg.[39] Although most corn pollen is dispersed downward from the tassel in the adjacent rows, within 6–15 m of the donor plant, some pollen may be carried by wind for considerable distances.[32,33,38,40] Ma *et al*.[40] measured 82% outcrossing in the immediately adjacent row, but the level of outcrossing dropped to <1% at 28 m downwind. Several studies have measured gene flow via pollen in corn, and in all cases there was a sharp reduction in pollen dispersal as the distance from the pollen source increased (see Halsey *et al*.[32] for references).

Corn hybrid seed production is particularly vulnerable to cross-pollination because of the lower pollen grain:silk ratio in the field.[41] Therefore, reproductive isolation (i.e. in space or time) of seed fields is required to ensure genetic purity of the hybrid produced, whether it is GR or non-GR. Spatial isolation is one of the most effective ways for reducing pollen adventitious presence.[33,42] Although isolation distances can reduce gene flow via pollen, it is unlikely that they will result in complete containment.[3] The recommended isolation distance between two corn fields for seed production is 185–200 m.[20,42] Even though current isolation practices in hybrid seed production often achieve the goal of ≥99% genetic purity, levels of outcrossing as high as 21% were observed within 36 m of the field edge, and up to 15% at the field midpoint (200 m from the pollen source).[41] Corn pollen is usually viable for 2–24 h under favorable conditions.[33,40,42] Therefore, synchronization of pollen dispersal and silking (nicking) is critical for the occurrence of pollen-mediated gene flow.[40] Temporal isolation or separation is used to disrupt or prevent nicking to maintain genetic purity.[40] Isolation in time is typically more effective than isolation in space as a reproductive barrier.[3] However, it is important to note that temporal isolation is actually dependent on accumulation of heat units and cannot be considered as chronological time. Halsey *et al*.[32] found a level of ≈80 growing degree units, with base 10 °C, is required for temporal isolation to be effective.

Time and distance together are expected to provide sufficient confinement of the *CP4 EPSPS* gene in corn, but sometimes an increase in temporal isolation is used to decrease spatial isolation.[32] Halsey *et al*.[32] evaluated the relationship of spatial and temporal isolation in reducing pollen-mediated gene flow. An increase in temporal isolation reduced the distance required to achieve reproductive isolation. While <0.01% outcrossing was measured at 500 m when source and receptor flowered at the same time, the

same level of confinement was achieved at 62 m or less when a 2 week temporal separation was used.[32]

Because it is expected that adventitious pollen would pollinate more plants on the borders of a field, harvesting the outside rows separately from the rest of the field should lead to higher genetic purity.[40,41] Border rows around the source or recipient corn field can reduce cross-fertilization levels more effectively than an isolation distance of the same length.[33,43] Although border rows could help reduce adventitious presence of the *CP4 EPSPS* gene, a large number of border rows (>12) alone is not enough to prevent outcrossing.[41] The limited pollen produced in hybrid seed production fields may not be sufficient to protect the interior of the fields from adventitious pollen sources.[41]

In the previously mentioned 2004 UCS study, where six non-GE corn hybrids were analyzed, transgenes were found in 50% of the samples at levels from 0.1 to 1%.[23] No information on the mode of contamination is available. However, it would be reasonable to assume that both pollen and seed commingling have contributed to the adventitious presence, based on the outcrossing nature of corn and the large number of hectares produced in the USA.

2.4 Sugarbeet (*Beta vulgaris*)

Glyphosate-resistant sugarbeet was deregulated in 1998 in the USA, but not commercialized because of reluctance from sugar companies to purchase the sugar from GE sugarbeet.[6] In 2006 there was a move by several sugar companies to accept GE sugarbeet. Widespread commercial production of GR sugarbeet in the USA is planned for the 2008 growing season. In 2006, conventional sugarbeet was produced for roots (i.e. sugar) on approximately 0.53 million ha in the USA [USDA-NASS (http://www.nass.usda.gov/QuickStats/index2.jsp)]. Nearly 100% of the sugarbeet seed production in the USA is in the Willamette Valley of Oregon. Sugarbeet seed production area ranges from 1000 to 2500 ha. In 2006, fewer than 1500 ha were harvested.

Sugarbeet is an outcrossing, wind-pollinated species. Although sugarbeet is biennial, it is grown as an annual for both seed and root production. When sugarbeets are grown for roots, they are harvested for processing before the plants bolt and flower. Occasionally, there will be some plants that bolt and set seed before harvest; however, gene flow via pollen or seed in root production fields generally is not an issue. Sugarbeet does not produce feral populations in the USA but does have two compatible relatives, *B. macrocarpa* Guss. and *B. vulgaris* ssp. *maritima* (L.) Arcang., in California (USDA plant data base). Bartsch and Ellstrand[44] reported hybridization and introgression of *B. vulgaris* alleles in an accession of *B. macrocarpa,* which is a widespread weed in sugarbeet fields in the Imperial Valley, California. They also suggested that *B. vulgaris* can hybridize with *B. vulgaris* spp. *maritima*. Therefore, there is potential for the *CP4 EPSPS* gene to persist outside cultivation.

Sugarbeet seed is produced using a hybrid system [American Crystal Sugar Company (http://www.crystalsugar.com/agronomy/bs.new/producingseed.asp)]. Rows of pollen donor and pollen receptor plants are planted, and seed is harvested only from the pollen receptor plants. Sugarbeets grown for seed are sown in late summer and receive enough vernalization to bolt and set seed the next summer, thus eliminating the need for two growing seasons.

Isolation distances for the production of non-GE sugarbeet stock and certified seed are 1500 and 1000 m respectively, and the distances increase to 3000 and 2400 m from fields of red beet (*B. vulgaris* ssp. *vulgaris*) and Swiss chard [*B. cicla* (L.) Koch], two conspecific species that are also grown for seed in the Willamette Valley. However, as most of the sugarbeet seed is not certified, isolation distances are voluntary within the seed production industry. The two major sugarbeet seed companies have addressed the issue of pollen flow from GR sugarbeet to other compatible crops by requiring growers to increase the isolation distance to 4800 and 8000 m respectively (Burt G and Standard JR, 2007, private communication). Still, it is possible for a seed-producing field to be planted closer than the recommended isolation distances. In addition, sanitation protocols have been established to prevent physical transfer of GR pollen between fields. For example, crop advisors must wear clean coveralls to enter a GR field and must remove them after exiting the field.

In order to prevent seed commingling, seed producers are not allowed to grow conventional and GR sugarbeet seed on their farm in the same year. In addition, GR seed is cleaned and stored separately (Burt G and Standard JR, 2007, private communication). Seed shattering occurs during harvest, and volunteer plants need to be controlled in subsequent crops.

2.5 Canola (*Brassica napus*)

Canola can be either *B. napus* or *B. rapa* L. (formerly *B. campestris* L.). In North America, most of the canola grown, and all GR canola, is *B. napus*. Glyphosate-resistant canola was introduced in Canada in 1995 and was overwhelmingly accepted by growers. In 2005, less than 10 years after its introduction, over 50% of the canola produced in Canada was glyphosate resistant.[45] Glyphosate-resistant canola was deregulated in the USA in 1999. By 2004, GR canola was grown on about 70% of the 320 000 ha grown in North Dakota [NDSU (http://www.ag.ndsu.edu/weeds/Surveys.htm)]. The North Dakota production accounts for 75% of the total hectares planted in the USA.

Canola is an annual, self-fertile and outcrossing species that is both insect and wind-pollinated and has the potential to establish outside cultivation. Outcrossing rates as high as 47% have been reported.[46] Canola pollen dispersal was found to range from a few

meters to 1.5 km.[47] The majority of pollen moved less than 10 m, and the pollen levels decreased with increasing distance from the pollen source. Pollen movement depended on wind direction and speed, surrounding vegetation and on topography.[48,49] Under controlled conditions, canola pollen can remain viable for up to 1 week,[50] but under dry and hot field conditions viability may be much shorter. Bees are known to pollinate canola. Most bees forage close to the hive, but there are reports of movement up to 4 km.[49,51] Because loose pollen grains can be picked up in a hive, a 4 km flying distance could result in pollen being moved 8 km. In Canada, gene movement between two GE lines was found at 800 m, which was the limit of the study.[52] In a Canadian field, volunteer canola plants were identified that had transgenes for both GR and GE glufosinate-resistant canola.[53]

Although canola does not generally survive outside cultivated fields or in undisturbed habitats, it does survive in areas adjacent to agricultural sites, roadsides and field edges.[54] The occurrence of GR canola along railways and roadways in Canada was measured in 2005.[55] In Saskatchewan, 34% of 300 canola plants tested were GR; in British Columbia, 43% of 81 plants tested were GR. One GR hybrid between *B. rapa* and *B. napus* was identified. In the same study, GE glufosinate-resistant plants also were found.

Volunteer canola can be a significant weed problem in subsequent crops.[56,57] Kaminski[57] reported that volunteer canola was the fourth ranked weed in Manitoba. For 35 fields sampled, harvest seed loss ranged from 3 to 10% with an average of about 6% or 107 kg ha^{-1}.[58] There are about 300 seeds g^{-1}, so it is possible that 3 million seeds ha^{-1} could be returned to the field. In general, canola seedbanks decline quickly but may persist for several years.[59] Secondary seed dormancy, which allows seed to survive more than one season and produce a persistent seedbank, was found to vary by canola genotype.[59] In addition, Pekrun and Lutman[60] found that canola seeds survived longer when buried.

The *Brassica* genus includes crops and weed species, and the genetics and taxonomy of the genus are complex. A unique aspect of the *Brassica* crop group is that several crops with highly different morphologies were derived from the same species and are therefore highly interfertile.[61] Many studies have addressed gene flow via pollen from GE or conventional canola to weedy or wild relatives.[62–67] These studies do not address gene flow to the *Brassica* vegetable crops. When vegetable crops are mentioned, authors generally state that they are harvested before they flower, so gene flow is not a concern. This is true if the crops are being produced for human consumption, but not if they are being grown for seed production. International purchasers of *Brassica* vegetable seed crops have extremely low tolerances for any adventitious presence and zero tolerance for GE adventitious presence (Tichinin N, 2007, private communication). Growers currently maintain a *Brassica* weed- and crop-free zone with a 3.2 km radius around a vegetable seed field.

Cabbage, kohlrabi, cauliflower, broccoli, Brussels sprouts and kale originated from *B. oleracea* L. Canola and *B. oleracea* hybridization is not common; however, spontaneous hybrids have been found in the wild.[68] Canola and rutabaga are *B. napus*, while Chinese cabbage and turnip are both *B. rapa*. Crosses are common between *B. rapa* and *B. napus*, but levels of hybridization vary widely and hybrids are reported to have reduced fertility and seed set when compared with the parents.[62] Very low levels of hybridization between canola and wild radish (*Raphanus raphanistrum* L.) were reported in field studies.[69–72] Crosses of canola with radish (*R. sativus* L.) seed crops have not been studied. There is the potential for canola to cross with a compatible relative and form a hybrid that has the potential to cross with another *Brassica* species.[73] These compatible species are considered bridge-species that increase the potential for *CP4 EPSPS* gene flow.

In Canada, all but one of 25 certified seed lots of non-GE canola tested in 2002 had detectable levels of GE seed.[74] Six out of seven GE glufosinate-resistant certified seed lots tested contained the glyphosate resistance transgene. These results, obtained 7 years after the introduction of GE canola in Canada, provide strong evidence that it will be difficult to prevent *CP4 EPSPS* gene flow if a grower cannot be assured of the purity of seed being planted. The 2004 UCS study showed that conventional USA varieties frequently contained the glyphosate resistance transgene.[23]

2.6 Alfalfa (*Medicago sativa*)

Glyphosate-resistant alfalfa was the first perennial GE crop to be deregulated (June 2005) and to be commercially available in the USA. In 2006, GR alfalfa was seeded on 80 000 ha, representing 5% of the approximately 1.3 million ha seeded in the USA.[75] However, in March 2007, GR alfalfa became the first GE crop returned to regulated status after approval by USDA-APHIS. A permanent injunction prohibits further planting of GR alfalfa in the USA after March 2007 pending the completion of the USDA-APHIS Environmental Impact Statement and a decision on the deregulation petition.[76] The harvest, use and sale of already planted GR alfalfa forage may continue. The ruling's effect on the 2007 GR alfalfa seed production, approximately 8000 ha, remains unclear.[8,76]

Alfalfa is the world's most important forage crop, and now is the third most important crop in economic value in the USA, exceeded only by corn and soybeans.[77–79] Alfalfa is a perennial, mainly outcrossing, insect-pollinated crop. Although there are wild populations of *M. sativa* and its relatives in Spain and the area from the Near East to Central Asia,[80] no compatible wild relatives are known to exist in the USA.[78,81] However, feral alfalfa populations (i.e. domesticated plants growing outside fields) are common in areas of alfalfa cultivation. In a survey

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of 940 roadside sites in 47 counties in California, Idaho, Pennsylvania, South Dakota and Wisconsin, approximately 22% of the sites had feral alfalfa populations within 2000 m of cultivated alfalfa.[82] Non-GR (organic or conventional) alfalfa fields also would be receptive to introgression of the *CP4 EPSPS* gene.[82]

More than 8.9 million ha of alfalfa and alfalfa mixed hay were grown in 2005–2006 [USDA-NASS (http://www.nass.usda.gov/QuickStats/index2.jsp)]. Putnam[79] analyzed the alfalfa hay market and concluded that the vast majority of alfalfa is grown for uses or markets that accept other GR crops and are not highly sensitive to the presence of the transgenic trait. The exception would be the export markets, organic markets and some hay grown for horses, which are likely to be less than 5% of annual USA production. These more sensitive markets would require the coexistence of GR alfalfa with non-GR alfalfa in regions where the non-GR markets are important.[79] Some practices that could reduce adventitious presence of the glyphosate resistance trait in non-GR alfalfa hay are: using certified conventional seed that has been tested for the presence of the *CP4 EPSPS* gene, preventing excess flowering and seed set, controlling feral alfalfa near hay fields that could serve as a bridge for transferring the transgene, labeling non-GR hay and preventing mixing of hay lots.[79]

In the USA, alfalfa seed is produced primarily in the western states, on about 40 500 ha, of which 20% are GR.[78] Insect-mediated pollination is necessary for alfalfa seed production.[83] Therefore, control of pollen movement between seed fields is imperative for maintaining genetic purity, whether the alfalfa being produced is GR or not. One of the management practices used to ensure genetic purity is field physical isolation. Current isolation standards in the USA are 274 m for foundation seed and 50 m for certified seed production for fields of 2 ha or less, and 183 and 50 m for foundation and seed production, respectively, for fields larger than 2 ha.[78,84]

In a study that evaluated gene flow from hay and seed production alfalfa fields, gene dispersal via pollen beyond current isolation standards was reported.[81] In this study, as much as 22% of the seed tested from trap plants located at the border of the study, 1000 m from production fields, had the marker. In comparison, marker genes were detected at 200 m away from research plots, demonstrating that the size of the area planted to a GE crop will affect the extent of gene dispersal.[81] Seed production fields had 38% higher outcrossing rates than hay fields, but hay fields still had an outcrossing rate of >25%. Although the current isolation standards are enough to maintain variety purity within acceptable levels, the coexistence of GR and non-GR varieties may require different purity standards to meet specific market demands; therefore, there may be a need to increase isolation distances.

In a 3 year study, Fitzpatrick *et al.*[83] measured pollen-mediated gene flow from 0.4–0.8 ha GR alfalfa seed production plots using leafcutter bees. Gene flow decreased as distance from the pollen source was increased. In general, gene flow was <0.5% at 274 m. In one of the years, 0.003% gene flow was detected as far as 805 m from the pollen source, but no transgene was detected 1207 m from the source in any year. In California, movement of the GR gene from a 2.4 ha GR alfalfa plot into trap crops was detected at distances up to 4 km away from the pollen source. Pollen-mediated gene flow was <1.5% at 274 m and decreased to <0.2% at 1.5 km.[78] Two studies that evaluated gene flow from GR alfalfa hay fields to non-GR alfalfa seed fields when harvest was delayed (20–50% bloom) found that gene flow into the seed fields was around 0.2% at shorter distances than the current isolation standards and was 0.00–0.05% at 172 m (http://ucce.ucdavis.edu/specialsites/alf_seed/year.asp).

Maintaining the physical identity of the alfalfa seed lots to prevent seed admixture is critical to preserve genetic purity. Alfalfa seed is small, approximately 500 seeds g^{-1},[30] which may increase commingling between alfalfa seed lots. Hard seeds, which are common in alfalfa, may lie dormant for years before absorbing water and germinating.[85] Dormancy allows alfalfa seeds to persist in the seedbank and become volunteers in subsequent crops. The presence of volunteer transgenic alfalfa in non-GR fields could result in hay containing the *CP4 EPSPS* gene or in seed lots with GR seeds.

Although alfalfa is not usually considered to be vegetatively propagated, it can be propagated by stem cuttings, and alfalfa crowns can persist and regenerate new plants.[86] Alfalfa crowns moved by machinery within and between fields could result in *CP4 EPSPS* gene flow. While no studies were found regarding vegetative transgene flow in alfalfa, this avenue of gene flow needs to be addressed.

2.7 Creeping bentgrass (*Agrostis stolonifera*)

Glyphosate-resistant creeping bentgrass is still under USDA-APHIS regulated status. If GR creeping bentgrass is deregulated, it will be the first GE perennial grass to be commercially available. Creeping bentgrass is a controversial species to genetically engineer, because there is the potential of transgene escape not only through pollen and seed but also through vegetative propagules. Creeping bentgrass is a cool-season turfgrass, mainly used in golf greens and other playing fields where, if it is well managed, seed production is prevented.[87,88] Bentgrass seed is produced in Oregon, with about 3400 ha under production in 2005 [Oregon State University Extension Service (http://extension.oregonstate.edu/catalog/pdf/sr/sr790-05.pdf)].

Creeping bentgrass is a perennial, wind-pollinated, outcrossing, mainly self-incompatible, small-seeded species, which also reproduces vegetatively by stolons. Creeping bentgrass pollen grains are approximately 25.4 μm in diameter and weigh 52 ng on average.[89]

Pollen remains viable up to 2 h.[89,90] *Agrostis* is a cosmopolitan genus that has nearly 200 species distributed worldwide.[88,91] Creeping bentgrass is part of a complex of interpollinating, cross-compatible species with redtop (*A. gigantea* Roth) and other *Agrostis* spp. that readily cross when they are sympatric and result in interspecific hybrids with varying degrees of pollen fertility and seed set.[92,93]

While still a regulated event, GR creeping bentgrass seed production fields were planted in 2002 on 162 ha, within a 4500 ha seed production control area.[94] The control area was established near Madras, OR, which is >150 km away from the major grass seed production area in the Willamette Valley of Oregon. The control area was established because of concerns about transgene flow from the seed production fields to non-GE grass seed fields. Production practices were regulated and monitored to minimize transgene movement from the GR creeping bentgrass fields.

Based on seedlings screened in the greenhouse, Watrud *et al*.[95] reported the occurrence of pollen-mediated *CP4 EPSPS* gene flow to susceptible sentinel and resident creeping bentgrass plants at 21 and 8 km, respectively, from the control area perimeter. Resident redtop plants as far as 14 km from the perimeter of the control area produced GR seedlings as well.[95] In a follow-up study, Reichman *et al*.[96] reported the establishment of nine GR creeping bentgrass plants up to 3.8 km beyond the control area perimeter (0.04% of samples tested in 2004–2005), and suggested that the established plants resulted from both pollen-mediated intraspecific hybridizations and from seed dispersal.

We conducted surveys within and around the control area, beginning in 2003, to assess the presence and distribution of GR creeping bentgrass plants.[97] Transgene flow via pollen was found, beginning in 2003, and has been found in all subsequent surveys.[97,98,99] In 2006, 3 years after the GR creeping bentgrass source fields were taken out of production, 62% of 585 creeping bentgrass plants tested in the area carried the *CP4 EPSPS* gene,[97] and 0.012% of 49 351 seedlings produced from seed of glyphosate-susceptible plants were GR.[99] In addition to the established plants, these results show that the *CP4 EPSPS* gene is still being moved via pollen. Although GR creeping bentgrass plants were found in different locations each year, which suggests that gene flow occurred via seed, it is not possible to determine whether the *CP4 EPSPS* gene moved via pollen, seed or vegetative propagules.[97,99] These results demonstrate that the isolation requirements of 274, 200 and 91 m for foundation, registered and certified creeping bentgrass seed production, respectively,[84] are not enough to prevent *CP4 EPSPS* gene flow.

Creeping bentgrass has extremely small seeds, 13 500 seeds g^{-1},[30] and therefore the potential for gene flow via seed is high. Creeping bentgrass shatters easily at maturity, and seeds can remain viable in the soil. In a seed longevity study, after 4 years of burial, there was no difference in germination between GR and non-GR seeds (Mallory-Smith C, unpublished data). Creeping bentgrass is harvested in two steps. The crop is first swathed and it is allowed to dry on the field until it is ready to be threshed. During the time the crop is lying on the swaths, seeds and whole panicles can be moved by wind (personal observation). Then, once harvested, creeping bentgrass seed must be cleaned and conditioned before being sold. Seed commingling can occur in the cleaning facilities.

Gene flow via vegetative propagules of creeping bentgrass is likely to occur. In the past, vegetative planting of creeping bentgrass was used for establishing putting greens on golf courses.[15] Vegetative reproduction of creeping bentgrass and hybrids between creeping bentgrass and redtop was studied under greenhouse conditions (Dysart P and Mallory-Smith C, unpublished). Plants emerged from a single node of creeping bentgrass or the hybrids when buried at 15 cm. Node fragments withstood prolonged drying for 5 days at 20 °C and initiated roots and tillers after being submerged in water at 4 °C up to 60 days with and without supplemental oxygen. In addition, hybrids between creeping bentgrass and redtop have both rhizomes and stolons. In areas of seed production with irrigation canals and drainage ditches, plant fragments are easily moved from one site to another (personal observation). Machinery can move vegetative propagules from one field to another. Vegetative propagules left in the soil can result in an established plant in the following year, which makes eradication difficult. Gene flow via vegetative propagules must be taken into account with GR creeping bentgrass.

3 DISCUSSION

In summary, it is clear that not all GR crops have the same potential for gene flow. Both soybean and cotton have low risk of gene flow via pollen, and transgene escape to compatible relatives is not an issue in the areas where they are produced in the USA. However, pollen is the main dispersal route for transgenes in corn, and also is important in canola, creeping bentgrass, alfalfa and sugarbeet grown for seed. While corn and alfalfa have no compatible relatives in the USA, alfalfa can form feral populations. Canola and creeping bentgrass do have many widespread compatible relatives, and both can persist outside cultivation. In contrast, sugarbeet does not produce feral populations and only has compatible relatives in California.

Sugarbeet, canola, creeping bentgrass, soybeans and alfalfa shatter, and GR volunteers can be an issue. Although shattering is not a problem in corn, seeds or cobs left in the field have the potential to result in volunteers the following year. In addition, creeping bentgrass seeds and panicles can easily be moved by wind, contributing to gene flow. Because of the small seed size and the need to clean and process the seed, seed commingling during handling, transport and processing of creeping bentgrass is likely

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to occur and result in the adventitious presence of the *CP4 EPSPS* gene. Although they have larger seeds, seed commingling can also occur and result in transgene adventitious presence in alfalfa, canola, soybean, cotton and corn. Seed commingling appears not to be an issue in sugarbeet because there are only a few seed production companies and seed is grown on a small area. The companies also require identification and physical separation of seed lots.

Both alfalfa seed and crowns are long lived, so the presence of volunteers carrying the *CP4 EPSPS* gene due to seed or crowns left in the field is a potential problem. Stolons and rhizomes of intra- and interspecific hybrids also can contribute to gene flow via vegetative propagules in creeping bentgrass.

When transgene flow via pollen to other cultivars is an issue, it can be reduced by isolation in either space or time. Increasing the distance between fields can reduce transgene flow because most pollen remains close to the source. Modifying planting dates to prevent overlapping flowering times between fields may further reduce gene flow via pollen, at least for corn.[32] In alfalfa, the hay harvest date can be optimized to reduce pollen production and seed set.[78] Control of gene flow via pollen to non-agricultural sites is difficult because it requires that all compatible plants be found and removed. In addition, flowering times among species are unpredictable and often extended. Natural dispersal of pollen via wind and insects cannot be prevented or predicted, but can occur over considerable distances.[52,95,96]

Gene flow via seed can be reduced with altered production practices, but seed will still be lost during each step from planting to final use (Fig. 2). The end use of a crop impacts the probability and effect of gene flow via seed. For example, the goal for grass seed production is large volumes of high-quality viable seed that will be sold and planted at sites that are great distances, sometimes continents away, from the area of production. Examples of seed commingling continue to be reported.[23] Human error will continue to lead to seed commingling. However, commingling could be reduced by volunteer control, proper cultivar identification, cleaning of equipment, handling, processing and storage. Natural dispersal of seed via wind, water and animals contributes to gene flow and cannot be prevented.

Gene flow via vegetative propagules can also be an issue for GR crops such as alfalfa and creeping bentgrass. For example, glyphosate has been one of the primary herbicides used to remove alfalfa stands. Although alternative herbicides followed by cultivation also can be effective, there is the potential for persistence of unwanted GR plants in subsequent crops.[78] Therefore, vegetative reproduction must be taken into account when developing management plans for crops that reproduce via stolons, rhizomes, crowns, etc., irrespective of their outcrossing potential.

Gene flow is a natural phenomenon and is not unique or different for GE crops versus non-GE crops. Gene flow from GR crops has occurred and continues to occur. This is also true for other GE crops with other traits; but the number of GR crops and the frequency that they are used may increase the potential for gene flow. An issue with monitoring gene flow is that the location of the last pollen grain, seed or vegetative propagule is unknown. Gene flow cannot be prevented with the technology available today, but in many cases can be reduced. However, once released into the environment, a transgene will persist as long as there is no selection against it or fitness cost associated with it. The glyphosate resistance trait has not been shown to have a fitness cost.[4,100] Herbicide resistance transgenes have been shown to persist over time in *B. napus* by *B. rapa* hybrids without selection pressure, even though the hybrids themselves had a fitness penalty.[101]

Although the GR trait is not generally considered to have a negative environmental or human health impact,[4] the documented gene flow from GR crops provides a strong argument for a more critical evaluation of gene flow. Movement of transgenes for traits such as drought or salt tolerance could change the ecological amplitude of a species and present a risk to endangered species or fragile ecosystems. The potential for gene flow that could result in the adventitious presence of transgenes responsible for pharmaceutical or industrial products obviously requires more scrutiny by regulators. It would be irresponsible of regulators not to consider the GR crop gene flow data when formulating rules for the release of GE crops in the future.

Recent concerns of USA international trade partners regarding the presence of GE seed in non-GE seed lots have raised significant doubts as to whether current practices are enough to meet the market demands.[23,33,41,78] Based on the experience of gene flow from GR crops, zero transgene tolerance is unachievable; therefore, accepted tolerance levels are needed for coexistence of GE and non-GE crops.

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