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REVIEW

Herbicide-resistant genetically modified crop: assessment and management of gene flow

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Genetically modified (GM) crops have become a reality in our cropping system. The experiences with GM oilseed rape have shown that gene flow from a GM crop causes genetic contamination of non-GM crops and natural flora. This review summarizes technically available methods for gene flow assessment and proposes possible management methods. Methods for direct monitoring of gene flow include direct bioassay of plants and detection of phenotypic and molecular genetic markers contained in GM crops. A recent green fluorescent protein (GFP) marker technique can be powerful in monitoring gene flow as GFP inserted into a plant can be observed macroscopically under UV light. Appropriate analysis of data from direct assessment may give more useful information to mitigate gene flow. Observation with direct method provides real-time data and mathematical-statistical approaches may enable the long-term consequence to be predictable. Although an estimated gene flow is less than an acceptable level, gene flow must be maintained as low as possible with a systematic management. The management should be conducted stepwise; selection of gene flow-proof GM crops in the stage of development, risk assessment and regulation in the registration stage, cultural management, produce handling/transportation and a long-term monitoring in cropping stage. Promising methods for developing gene flow-proof GM crops include conferring cleistogamy and chloroplast transformation to mitigate pollen flow, and breeding non- or minimum shedding cultivars to mitigate seed dispersal. We strongly suggest that very high expression of a transgene or stacking multiple transgenes in the chloroplast could disturb the function of normal physiology, hence decreased performance of the GM crop. Before the approval of GM crops, proposed GM crops must go through the risk assessment. If the estimated risk of a GM crop exceeds an acceptable level, approval must be suspended. Once a GM crop is allowed for commercial release, additional efforts must follow, such as a continued long-term monitoring of the impact of GM crop cultivation, crop and herbicide rotations, GM crop-suited cultural practices, 'right-time' harvest, and all necessary gene flow-preventive practices. Such a systematic management incorporating various methods for the stages of GM crop utilization will minimize the risk of gene flow.

Keywords: chloroplast transformation, gene flow, GM crop, herbicide-resistance, risk assessment

INTRODUCTION

Despite concerns on risks arising from genetically modified (GM) crops, GM crops have become a reality in our agro-ecosystem and diets. Various products from them are now being sold in supermarkets. Many efforts have been made to define risks resulting from introducing GM crops and to classify them (e.g. Rieger *et al.* 1999; Kwon and Kim 2001). In the case of herbicide-resistant GM crops, the major risk on environment is the gene flow via pollen and seed, resulting in contamination of nearby non-GM crop with the transgene, establishment of herbicide-resistant volunteer weeds in

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the crop field and nearby non-cropland, and disorder and contamination of a genetically well-balanced plant kingdom with alien genes. A recent review by Kwon and Kim (2001) has shown that the risk of gene flow from GM crops is well evidenced in oilseed rape (OSR), which self-pollinates normally, but outcrosses in part (less than 10%), and concluded that substantial gene flow will be also possible in inbreeding major crops such as rice, wheat and barley. For instance, glyphosate-resistant OSR was found in a non-GM OSR field adjacent to a field with a glyphosate-resistant GM OSR cultivar in Canada (Downey 1999), implying that a resistant gene from the GM cultivar travelled to the field planted with a non-GM cultivar and introgressed into the non-GM cultivar. Another possible and immediate risk is herbicide-resistant GM crops becoming volunteer weeds. This genetic contamination of non-GM crops with GM crops or *vice versa* decreases their purity, which may cause economic loss.

Despite the agricultural benefit of GM crops, possible disadvantages should not be neglected. Even if the advantages of a GM crop outweigh its potential risk to agriculture, commercial release of the GM crop should be accompanied by appropriate risk assessment and management. A systematic monitoring of gene flow is the first step to keep transgenes contained. Based on obtained data, a potential gene flow can be estimated. Reproduction biology and molecular genetics may help to recalculate the potential gene flow to give an actual gene flow. Once the actual gene flow is estimated, the next step is to establish a management strategy of gene flow. Thus, in this paper, assessment and management of gene flow from GM crops are reviewed with particular interests on new techniques to monitor and to prevent gene flow.

ASSESSMENT OF GENE FLOW

The imminent commercialization of GM crops requires accurate quantification of transgene movement via pollen or seed within realistic agricultural contexts (e.g. Thompson *et al.* 1999) during the research and development stage. Gene flow is measured in two ways: by direct and indirect methods (Gliddon 1999; Raybould & Clarke 1999). Direct methods involve the estimation of the parameters of dispersal distributions from the source (Gliddon 1999), while indirect methods involve the use of techniques developed in population dynamics and genetics theory to estimate rates of gene flow in natural population (e.g. Goudet *et al.* 1994). The direct methods only measure gene flow at the time of observation, whereas the indirect methods measure average amounts of gene flow, by reflecting the cumulative effects of temporal variation in the spatial distribution of dispersal and establishment over preceding years, including rare and unpredictable events (e.g. Slatkin 1985).

Direct monitoring

The most common direct method for estimating potential gene flow is the observation of pollen and seed movement (dispersal). Other direct methods use genetic markers to estimate actual gene flow. A simple method is to introduce or identify a plant in a population with a unique genetic marker (e.g. an isozyme allele) and to follow the appearance of the marker in the next generations (e.g. Latta *et al.* 1998). Gene flow and transgene persistence in the environment have been monitored largely using phenotypic (Manasse 1992; Luby & McNicol 1995), biochemical (Klinger *et al.* 1992) or molecular markers (e.g. Jørgensen & Anderson 1994). A more sophisticated approach uses markers to identify the fathers of half-sib families. If the markers are highly variable (e.g. microsatellites) and the number of potential fathers is relatively small, the father of each seed can be identified unambiguously (e.g. Dow & Ashley 1998). With the recent availability of green fluorescent protein (GFP) encoding genes, a tractable monitoring system is feasible and has been recently introduced (Stewart 1999). In this method, GFP in GM crops can be visualized non-destructively on a macroscopic scale using UV light. Green fluorescent protein from jellyfish has the unique characteristics of fluorescing green when excited with UV (360–400 nm) or blue (440–480 nm) light. Green fluorescent protein is inherited in progeny (Leffel *et al.* 1997) and is thus useful as a tag to mark transgenic plants *in vivo*. Many scientists (e.g. Pang *et al.* 1996; Stewart 1999) have demonstrated that whole-plant fluorescence with GFP is a powerful tool. In the case of a GM crop resistant to a herbicide, gene flow to other plants can be monitored by treating a suspicious plant with herbicide. If the plant survives at the full dose of the herbicide while a known susceptible plant dies, the plant contains a herbicide-resistant gene escaped from herbicide-resistant GM crop. For diagnosing herbicide resistance in weeds, many simple and rapid methods have been developed including juvenile plant, tiller and stem node tests (Kim *et al.* 2001), so these rapid methods would be useful to measure gene flow.

Data analysis

Although much information can be collected from monitoring the gene flow from GM crops, the absence

of appropriate analysis of the data makes it virtually useless for the purpose of risk assessment (Gliddon 1999). Comparisons based on the regression and modeling approaches are informative, especially for risk assessment of GM crops, in that they incorporate the effects of scale and inter-population distance (Raybould & Clarke 1999). Pollen or seed dispersal was assumed to follow a bivariate normal distribution (Wright 1943; Haldane 1948), but that from source plants has been found to be strongly leptokurtic (e.g. Levin & Kerster 1974), leading to an exponential power function (Kareiva *et al.* 1994). Rather than use of this essentially descriptive distribution, Lavigne *et al.* (1996) and Tufto *et al.* (1997) have proposed using methods based on a consideration of Brownian motion in three dimensions to describe pollen deposition. Under some conditions such as wind strength varying in direction during an experiment, this mechanistic method gives a better fit than the descriptive, exponential power function (Tufto *et al.* 1997). For insect-pollinated species, the crop species itself is unlikely to be a sufficient descriptor of expected pollen movement. Factors including the type and density of surrounding vegetation, flowering stage of other vegetation and meteorological conditions are likely to influence the distance that pollen is carried. Such variability needs to be taken into account if a risk assessment is carried out prior to the possible release of GM crops. A model system called GeneSys was developed to evaluate the influence of cropping systems on transgene escape from GM OSR to volunteer OSR in time and space (Colbach *et al.* 1999). Input variables of this model are the regional field pattern, crop succession and cultivation techniques. This system was possible to identify low-gene-flow cropping systems or the minimum distance between OSR plots needed to avoid contamination of the harvest product.

Seed bank and dormancy

There are not many data available on the long-term behavior of seed-bank of crops under arable conditions, so the only way to study seed-bank dynamics of GM crops over a long period of time is by modeling. Pekrun *et al.* (1999) simulated temporal change of seed-bank of OSR, beans and linseed in a rotation system at varying levels of volunteer control. In this model, the amount of seed loss and the level of volunteer control are important input variables. Gene escape in time via the soil seed-bank is only one aspect that needs to be considered in risk assessments of GM crops. More complex models such as the model by Colbach & Meynard (1996) are necessary to incorporate complicated effects of agronomic practices and ecological factors on gene flow.

Long-term impact

It is clear that a priority must be given to the risk assessment of GM crops particularly with an emphasis on gene flow prior to their commercial release. Detection of gene flow at any particular site is difficult; gene flow is likely to occur across a large area, depending on a complex set of local events (Keeler *et al.* 1996). As it takes a long time for the consequence of gene flow to be apparent, long-term assessment using only direct methods seems impractical. Moreover, there is the possibility of getting contrasting estimates: a higher estimate of gene flow by direct methods compared with indirect methods (e.g. Rasmussen & Brodsgaard 1992) or *vice versa* (Campbell & Dooley 1992). Therefore, if possible, it is desirable to use both types of method (Raybould & Clarke 1999). Observation with direct method provides real-time data and a mathematical-statistical approach based on preknowledge of gene flow can simulate long-term consequence. Thus the combination of these two can be a means of determining if the risk in question is acceptable or not.

Need for study on out-crossing and volunteering in agro-ecosystem

Sugar beet is an out-crossing crop. When GM sugar beets bolt, gene flow to non-GM beets causes trouble. This was expected and assessed using male-sterile beets (Champolivier *et al.* 1999). While OSR is known as a self-pollinating crop with an out-crossing rate of less than 10%, the experience with GM OSR, as research has progressed, has indicated that more wild relatives are capable of producing hybrids in the field than was thought when the early risk assessments were prepared (Downey 1999). How much do we know about the reproductive biology of donor (GM crop) and recipients (non-GM crop and feral plants)? Already, numerous herbicide-resistant weeds are making trouble. Some of the herbicide-resistant weeds may contribute to backward gene flow to related crops. Related to possible gene flow in the agro-ecosystem, we have recently made an extensive literature survey on information on the mode of pollination of troublesome herbicide-resistant weeds of the world. The Weed Science Society of America (2001) reports that 154 weed species are herbicide-resistant, and 248 biotypes show unique resistances. The search is still ongoing, however: 36 species are not known for their modes of pollination, and 28 species are not known even for their number or ploidy of chromosomes, among 73 herbicide-resistant weed species selected for study on their modes of pollination (Table 1). The

Table 1. List of some important herbicide-resistant weeds

Weed species	Pollination (self-/cross-)	Chromosome number and polyploidy	Estimated max. area infested (ha)	Incident situation	Resistant herbicide
Alopecurus myosuroides		2n=14	20 985	Wheat, sugar beet, roadsides	Clodinafop, fenoxaprop, isoproturon, chlorotoluron, cycloxydim, diclofop, imazamethabenz, atrazine, chlorsulfuron methabenzthiazuron, pendimethalin
Amaranthus hybridus	Interfertile within the genus	4X, 2n=32	66 104	Corn, soybean, cropland, vegetables	Nico-, primi- and thifen-sulfuron, atrazine, simazine, chlorimuron, flumetsulam, imazaquin, imazethapyr
Amaranthus lividus	Interfertile within the genus		225	Corn, vegetables, vineyards, cabbage, lettuce, onion	Atrazine, paraquat, simazine, imazethapyr
Amaranthus palmeri	Interfertile within the genus		90 062	Cotton, alfalfa, corn, sorghum, soybean	Chlorimuron, trifluralin, imazethapyr, pyrithiobac, atrazine, diclosulam, imazaquin
Amaranthus powellii	Interfertile within the genus	4X, 2n=34	41 504	Corn, wheat, cropland, soybean, carrot, orchards, mint	Atrazine, metribuzin, imazethapyr, linuron, monolinuron, cyanazine, terbacil, prometryn, terbutryne, imazaquin
Amaranthus retroflexus	Interfertile within the genus	4X, 2n=34	66 679	Corn, cropland, sugar beet, soybean, vineyards, orchards, grain sorghum, roadsides, railways, vegetables	Atrazine, metribuzin, simazine, linuron, primisulfuron, cloransulam, imazethapyr, terbacil, cyanazine, prometryn, fenuron, terbutryne, chlorsulfuron, imazaquin, chlorimuron, imazamox, thifensulfuron
Amaranthus rudis	Interfertile within the genus		862 171	Alfalfa, corn, grain sorghum, soybean, cropland	Chlorimuron, flumetsulam, imazamox, imazaquin, atrazine, imazethapyr, halo-, nico-, primi-, pro- and thifen-sulfuron
Ambrosia artemisiifolia		2n=36	8 500	Corn, soybean	Primi-, halo- and pro-sulfuron, cyanazine, cloransulam, chlorimuron, imazethapyr, atrazine, simazine, imazaquin, imazamox
Avena fatua	Self-; cross-: <2%	6X, 2n=42	1 786 177	Wheat, lupin, barley, cropland, cereals, oilseed rape, sugar beet	Iso-, sulfo- and rim-sulfuron, fenoxaprop, diclofop, fluazifop, haloxyfop, triallate, clodinafop, tralkoxydim, difenzoquat, clethodim, sethoxydim, imazamox, flamprop, pronamide, imazamethabenz
Avena sterilis	Cross-	6X, 2n=42	8 134	Clover, wheat, cereals	Diclofop, fluazifop, fenoxaprop
Avena sterilis var. *ludoviciana*	Cross-	6X, 2n=42	607	Wheat	Diclofop
Bidens pilosa			4 047	Soybean, coffee	Pyrithiobac, chlorimuron, imazaquin, imazethapyr, nicosulfuron, atrazine
Bidens tripartita				Corn	Atrazine
Brassica campestris	Cross-	2X, 2n=20	202	Corn	Atrazine
Brassica tournefortii		2X, 2n=20	202	Wheat	Chlorsulfuron, atrazine
Bromus diandrus		8X, 2n=56	2	Cereals, pastures	Haloxyfop, atrazine
Bromus tectorum	Self-	2X, 2n=14	42	Corn, wheat, orchards, kentucky bluegrass	Atrazine, chlorotoluron, primisulfuron, sulfosulfuron, simazine
Chenopodium album	Self-	4X/6X, 2n=36/54	225 694	Corn, potato, soybean, cropland, sugar beet, roadsides	Atrazine, metribuzin, cyanazine, lenacil, prometon, simazine, terbuthylazine, terbutryn, linuron, paraquat
Chenopodium ficifolium			4	Corn, vegetables	Atrazine
Commelina diffusa				Sugarcane	2,4-D
Cyperus difformis		2n=32	40 872	Rice	Bensulfuron
Conyza (*Erigeron*) *canadensis*			49 213	Corn, soybean, forest, vineyards, nurseries, orchards, peach, pastures, roadsides	Atrazine, paraquat, linuron, chlorsulfuron, simazine

Table 1. (*cont.*)

Weed species	Pollination (self-/cross-)	Chromosome number and polyploidy	Estimated max. area infested (ha)	Incident situation	Resistant herbicide
Conyza sumatrensis			4 110	Cropland, orchards, vegetables, tea, railways, roadsides	Diquat, paraquat
Daucus carota	Cross-	2X, 2n=18	20	Cropland, roadsides	2,4-D
Digitaria sanguinalis		2n=36	22	Corn, carrot, orchards, cropland, vegetables, onion	Fluazifop, haloxyfop, sethoxydim, atrazine
Echinochloa colona	Self-	6X, 2n=54	8 134	Rice	Propanil, fenoxaprop
Echinochloa crus-galli	Self-	4X/6X, 2n=36/54	830 654	Rice, corn, cropland, orchards	Atrazine, butachlor, cyanazine, simazine, pendimethalin, propanil, quinclorac, thiobencarb
Echinochloa phyllopogon	Self-	4X, 2n=36	8 094	Rice	Fenoxaprop, thiobencarb
Eleusine indica		2X, 2n=18	53 159	Cotton, cropland, vegetables, orchards, golf course, industrial sites	Imazapyr, fluazifop, propaquizafop, paraquat, glyphosate, trifluralin, pendimethalin
Erigeron philadelphicus	Cross-		4 047	Cropland, orchards, railways, roadsides	Paraquat
Fimbristylis miliacea		2n=10	40	Rice	2,4-D
Hordeum glaucum		2X, 2n=14	243	Alfalfa, cereals	Paraquat, diquat
Hordeum leporinum		4X/6X, 2n=28/42	42	Alfalfa, pastures	Paraquat, diquat, fluazifop
Ischaemum rugosum			40	Rubber, vegetables	Paraquat
Kochia scoparia	Cross-	2X, 2n=18	1 090 317	Corn, wheat, barley, cropland, railways, roadsides, cereals, industrial sites	Chlor-, met-, thifen-, nico-, pro-, rim-, sulfo-, triflu-, and tria-sulfuron, atrazine, tribenuron, imazapyr, cyanazine, imazethapyr, dicamba, sulfometuron
Lactuca serriola	Interfertile within 4 spp. (2n=18)	2X, 2n=18	81 179	Wheat, cropland, cereals	Triasulfuron, chlorsulfuron, metsulfuron
Lindernia attenuata			4	Rice	Bensulfuron
Lindernia procumbens			40	Rice	Bensulfuron, pyrazolsulfuron
Lolium multiflorum	Cross-	2X/4X, 2n=14/28	94 493	Wheat, oilseed rape, cereals, roadsides	Diclofop, sethoxydim, sulfomethuron
Lolium rigidum	Cross-	2X, 2n=14	>996 105	Barley, oilseed rape, wheat, cereals, cropland, triazine-tolerant Canola, orchards, railways, grain sorghum, apple, almonds, roadsides, pastures	Chlorsulfuron, diclofop, fluazifop, sethoxydim, tralkoxydim, trifluralin, chlorpropham, clomazone, ethalfluralin, imazapyr, metolachlor, metsulfuron, quizalofop, triallate, triasulfuron, amitrole, atrazine, simazine, glyphosate, clodinafop, haloxyfop, imazamox
Lolium perenne	Cross-	2X/4X, 2n=14/28	4 089	Wheat, railways, roadsides	Sulfometuron, chlorsulfuron, diclofop, fenoxaprop
Lolium persicum	Cross-	2X, 2n=14	20	Wheat	Diclofop
Monochoria korsakowii	Cross-		41 010	Rice	Bensulfuron, diquat, pyrazosulfuron, imazosulfuron, cyclosulfamuron
Monochoria vaginalis	Cross-		20	Rice	Bensulfuron
Panicum capillare		2X, 2n=18	4 047	Corn, cropland	Atrazine
Panicum dichotomiflorum	Self-	4X/6X, 2n=36/54	2	Corn, cropland	Atrazine
Poa annua	Cross-	4X, 2n=28	5 716	Nurseries, orchards, hops, pastures, turf, roadsides, railways	Amitrole, paraquat, atrazine, cyanazine, prometryn, ethofumesate, diuron, pendimethalin, prodiamine
Polygonum aviculare			1	Corn, cropland, apple	Amitrole, atrazine

Table 1. (*cont.*)

Weed species	Pollination (self-/cross-)	Chromosome number and polyploidy	Estimated max. area infested (ha)	Incident situation	Resistant herbicide
Polygonum lapathifolium			4 087	Corn, cropland, railways	Atrazine, cyanazine, lenacil, prometryn, terbuthylazine, terbutryn
Polygonum persicaria			4 451	Corn, railways	Atrazine, cyanazine, lenacil, prometryn, simazine, terbutryn
Portulaca oleracea			202	Carrot	Atrazine, linuron
Raphanus raphanistrum	Cross-	2X, 2n=18	283	Cereal, wheat, lupins, cropland, triazine-tolerant canola	Chlorsulfuron, metosulam, diflufenican, atrazine, simazine
Sagittaria montevidensis			44 535	Rice	Ben-, ethoxy-, met- and pyrazo-sulfuron, bispyribac, cyclosulfamuron
Scirpus juncoides			40	Rice	Bensulfuron
Scirpus mucronatus		4X, 2n=44	44 515	Rice	Azimsulfruon, bensulfuron, cinosulfuron, ethoxysulfuron
Senecio vulgaris		4X, 2n=40	16 497	Corn, nurseries, mint, roadsides, vineyards, vegetables, asparagus	Simazine, atrazine, cyanazine, lenacil, prometryn, terbuthylazine, linuron, bromoxynil
Setaria faberi	Self-		526	Corn, soybean, cropland, carrot, onion, sweet corn	Atrazine, fluazifop, sethoxydim, clethodim, fenoxaprop, quizalofop, imazethapyr, nico- and primi-sulfuron
Setaria glauca	Self-	2X/4X, 2n=18/36	4 087	Corn, cropland	Atrazine, cyanazine, simazine
Setaria lutescens			2	Soybean	Imazethapyr
Setaria verticillata		2X/4X, 2n=18/36		Corn	Atrazine, nicosulfuron, primisulfuron
Setaria viridis	Self-	2X, 2n=18	1 663 320	Wheat, barley, oilseed rape, cropland, flax, corn, sunflower	Ethalfluralin, tralkoxydim, trifluralin, diclofop, atrazine, fenoxaprop, atrazine, sethoxydim, imazamox
Setaria viridis var. *major*				Corn	Atrazine
Setaria viridis var. *robusta-alba*			20	Corn, soybean	Imazethapyr, nicosulfuron, primisulfuron
Sinapsis arvensis		2n=18	546	Oilseed rape, cropland, wheat, barley, soybean	2,4-D, dicamba, MCPA, metribuzin, chlor-, ethanmet-, met- and thifen-sulfuron
Solanum americanum	Self-; cross-: <17%	2X, 2n=24	4 047	Tomato	Paraquat
Solanum nigrum	Self-; cross-: <1.4%	6X, 2n=72	8 177	Roadsides, vegetables, orchard, pastures, corn	Atrazine, paraquat, simazine
Solanum ptycanthum	Self-	2X, 2n=24	445	Soybean	Imazamox, imazethapyr
Sorghum bicolor	Cross-	2X, 2n=20	4 047	Corn, cotton	Primisulfuron, nicosulfuron
Sorghum halepense	Cross-	2X/4X, 2n=20/40	4 492	Soybean, cotton, cropland	Fenoxaprop, fluazifop, quizalofop, sethoxydim, clethodim
Sorghum sudanese	Cross-	2X, 2n=20	4 047	Soybean	Fluazifop, haloxyfop
Sphenoclea zeylanica			2	Rice	2,4-D
Stellaria media			4 051	Cereals, wheat, barley, corn	Chlorsulfuron, atrazine, tribenuron
Xanthium strumarium			65 821	Cotton, soybean, corn	Imazaquin, imazethapyr, cloransulam, DSMA, MSMA, chlorimuron

Compiled from various sources: Weed Science Society America (2001), AGRICOLA Database (1970–2000), AGRIS (1975–1999), CAB CD-ROM Database (1993–1998), Fischer *et al.* (2000), Frankel & Galun (1977), Holm *et al.* (1997), Hubbard (1984), Itoh (2000), Matsuo (1989), McWhorter (1989), Ogg & Rogers (1989), Smartt & Simmonds (1995), Takematsu & Ichizen (1987) and Valverde *et al.* (2000).

out-crossing rate of a species may differ by ecotype, and even by the growing season and environmental conditions for an ecotype. Hence, presently available reports on out-crossing rates of weed species may serve only as a guide to work on gene flow problem in a particular location or region and cropping system. Our concern also has to be extended to cereals such as rice, wheat and barley, known as inbreeding crops. As shown in our previous review (Kwon & Kim 2001), barley and wheat can cross with their respective relatives, with the maximum out-crossing rate of approximately *ca*. 10%, while rice can cross with weedy rice and wild types at 1–2%. Pyon *et al*. (1998) and Watanabe *et al*. (1998) showed that weedy rice occurred much more and easily in dry direct-seeded rice fields than in flooded direct-seeded rice fields, suggesting that favorable conditions for rice to become weedy is more important than the extent of outcross. No systematic approach has been made to assess the risk of GM cereal crops including rice and wheat.

Social requests

Hill (1999) working at Green Alliance has emphasized the points for constructing a better monitoring regime to meet the concerns expressed by some influential groups. He has addressed 12 questions for development of a sound monitoring protocol: What kind of gene flow do we want to monitor? What is the frequency of gene flow? How much do we know about potential recipients of introduced genes? What is the distance over which we want to monitor? What should be the size of the sample of wild plants to be sure of confirming assumptions about frequency of hybridization? Are there adequate testing techniques and how practical are these to carry out on a large scale? What are the valid measurements of fitness of the plant having the introduced gene introgressed? Are the base-line data established about species diversity and levels of populations before there is any gene flow? What would be the non-target effects in a food-chain and are there base-line data available, in particular for insect-resistance genes? What is the measure against probable multiple stacking of different introduced genes? How much consultation will there need to be with neighboring farmers to ensure access to the crop to be monitored? How long should it be monitored to obtain a creditable conclusion on the long-term effects of gene flow such as enhanced fitness? Also important are dormancy characteristics of the crop×weed hybrid which will influence how long seeds containing transgenes can survive in the seed bank.

MANAGEMENT OF GENE FLOW

Despite the persisting risk of gene flow and its predictable impact on the environment, the development and utilization of herbicide-resistant GM crop have been already profound and become a global reality. The consequences of gene flow become apparent through a sequence of events: hybridization, introgression, adaptation and then dispersal (Jordan 1999). Appropriate mitigation measures at each stage can reduce risks. These may consist of development, registration and cultural practice phases, and none of them should be neglected. In this review, practical approaches are discussed.

Development of gene flow-proof GM crops

One of the principal mitigation measures would be appropriate selection of target crops. A species with high potential to out-cross or with many sexually compatible wild relatives may be avoided or be obliged with more extensive monitoring of gene flow. Presently, choosing target crops is primarily based on biotechnological availability and potential profit to the developer, overlooking possible environmental risk. Oilseed rape-weed hybrid seeds were reported under field condition (Jørgensen *et al*. 1996) and introgression of the glufosinate ammonium resistant trait from OSR to *Raphnus raphanistrum* in experimental conditions was also reported (Chèvre *et al*. 1997). Phosphinothricin-tolerance was detected in the hybrid *Brassica rapa*× *Brassica napus* and furthermore the trait was consistently detected in their backcrosses (Metz *et al*. 1997), which implies eventual introgression into related community. Because weedy species produce large amounts of seeds, small leakage may result in fast-multiplying gene contamination. Fertilization within same species and hybridization between related species can lead to introgression of transgenes into ecosystems (Darmency 1994; Ellstrand *et al*. 1996). For preventing gene pollution via pollen dispersal, three methods, namely male sterility, constitutional cleistogamy and maternal inheritance, may be exploited through genetic engineering and conventional breeding.

Pollen-flow cut-down

Localized expression of specific genes, such as RNases, can prevent pollen formation and produce male-sterile plants (Mariani *et al*. 1990). However, male sterility is only possible in those crops where the product is neither seed nor fruit that requires fertilization. And additional planting of non-GM plants is required to provide pollen for the male-sterile GM crops.

Cleistogamy is quite a common phenomenon in cultivated plants, being found in 29 families of plant (Lord 1981), and in about 70 genus of grasses (Connor 1979). Cleistogamic flowers need not to open at all to complete fertilization, and are characterized by a reduction in number and size of floral parts such as stamens, and by modifications of the perianth (Frankel & Galun 1977). Cleistogamy is controlled genetically in some varieties of sorghums, barley, wheat, oat, and rice (Sethi & Chhabra1990; Connor 1979; Merwin *et al*. 1981; Chhabra & Sethi 1991; Kurauchi *et al*. 1993). Recently, Won *et al*. (1998) have found a useful cleistogamous rice line. Spikelets of the cleistogamous line did not show anthesis at all during pollination and fertilization. The cleistogamy was caused by lack of lodicules in the floret, and was expressed stably under different conditions of temperature, day length and fertilizers. A single recessive gene was responsible for the expression of cleistogamy. The cleistogamy character did not affect other agronomic characters such as panicle length, number of panicles, days to heading and grain fertility, except for culm length, which was greater in cleistogamous plants of segregating populations.

In a majority of crop plants, plastid genes are inherited uniparentally in a strictly maternal fashion (Smith 1989). Although pollen from plants with maternal plastid inheritance contains metabolically active plastids, the plastid DNA itself is lost during the process of pollen maturation (Nagata *et al*. 1999; Pyke 1999) and hence is not transmitted to the next generation. Consequently, a trait introduced by genetic engineering into chloroplast genome would not be unintentionally transferred to sexually compatible relatives of the crops (Daniell *et al*. 1998). Furthermore, the plastid chromosome-encoded protein would not be produced in the pollen and thus would not affect insects that feed on pollen or pollen-coated plant tissues (Bogorad 2000). Plant genetic engineering via the nucleus is a mature technology that has been used very productively for research and commercial biotechnology. By contrast, the ability to introduce foreign genes at specific location in a chloroplast chromosome has been acquired relatively recently (Bogorad 2000). Since the first conclusive demonstration of stable introduction of cloned DNA into the *Chlamydomonas* chloroplast by Boynton *et al*. (1988), technical developments in plastid transformation and advances in our understanding of chloroplast gene expression have been tremendous. Current transformation technology, however, still limits much of the agronomic and industrial application of plastid expression. Efficient selection and segregation to the homoplasmic state has proven to be a limiting factor (Heifetz 2000). In fact, reliable and efficient plastid transformation and regeneration of fertile plastid transformants have been restricted to tobacco and potato (Heifetz 2000). Excellent reviews on details of these progresses are available (Hager and Bock 2000; Heifetz 2000).

Daniell *et al*. (1998) expressed a wild type petunia 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) as a dicistron downstream of *aadA* in tobacco plastids and obtained homoplasmic plants capable of surviving on 10 times lethal concentrations of glyphosate. International patent application WO 00/03022 describes homoplasmic plastid transformants of tobacco that are resistant to high levels of glyphosate by virtue of expressing either the EPSPS gene from *Agrobacterium tumefaciens* strain CP4, EPSPS from *Pseudomonas* strain LBAA, or the *AroE* gene from *Bacillus subtilis*. WO 00/03022 also describes transformation of tobacco plastids with constructs containing the *ctrl* gene encoding phytoene desaturase from *Erwinia carotova* and the *bxn* gene encoding a bromoxynil-specific nitrilase from *Klebsiella pneumoniae* to yield homoplasmic transformants tolerant to norflurazon and bromoxynil, respectively. McBride *et al*. (1994) successfully expressed the *Cry1Ac* protoxin gene under control of the 16S promoter with a chimeric ribosome-binding site derived from the tobacco *rbcL* gene and the 3′ untranslated region of the *rps 16* gene. This resulted in accumulation of *Bacillus thuringiensis* (Bt) toxin between 3 and 5% of total soluble leaf-protein in mature homoplasmic tobacco plants grown under greenhouse conditions. Although whole-plant insect resistance tests were not performed, leaf tissue was found to be highly toxic to target insect species.

Recently, leading molecular biotechnologists emphasize the advantages of plastid transformation over nuclear transformation in introducing a transgene to major crops. The most prominent advantages claimed are no outflow of pollen and a possibility of 10–50 times higher expression levels of a transgene necessary to insure the gene works effectively. Prevention of pollen flow could be achieved. However, expressing a transgene to a very high level or stacking a variety of transgenes in the chloroplast may mean that reduced quantity of amino acids are available for synthesis of ribulose-1,5-bisphosphate carboxylase which accounts for about 50% of soluble chloroplast-protein and 30% of soluble leaf-protein in healthy plants. Uptake of nitrogen by plant roots and its assimilation by leaves consume much photosynthetic energy. Nitrogen content in the leaf has often been a limiting factor for

increased photosynthesis and crop yield. If we assume for simplicity an allocation of 5% soluble leaf-protein for each transgene to the expression of Bt protoxin, glyphosate-resistance, glufosinate-resistance, and drought-tolerance, this totals 20% of soluble leaf-protein. Could the chloroplast function normally? Could we expect a normal crop yield? We suggest that this situation will surely bring about disturbances in the physiological function of the crop plant for yield formation.

Seed-dispersal cut-down

Seed shedding is another important factor for unintentional release of GM seeds resulting in volunteer weeds. Crops that have had a relatively short history of domestication, such as OSR, have contributed to the weed problem by shedding (Lutman 1993; Price *et al.* 1996). Utilization of less-shedding varieties as a recipient for transgenes may mitigate the problems. Shedding-proof cultivars will reduce the seed amount remaining in the field.

In the case of OSR, resistance to shedding could be introduced from related wild species such as *Brassica juncea* (Prakash 1988). In the case of the *indica* rice grain, shedding is a big potential problem for gene flow through seed dispersal. In South and Southeast Asian countries, traditionally easy shedding cultivars have been widely cultivated for easy threshing. *Indica* varieties are known to shed grains more easily than *japonica* varieties. The yield loss due to grain shedding was estimated to be 3–30% in India (Bhalerao 1930). Studies done by the International Rice Research Institute (IRRI) reported a 5–15% loss of the rice crop during harvesting and threshing in several South and Southeast Asian countries (Chandler Jr 1979). The field loss of grains during harvesting *japonica* cultivars is less than 1%. Kwon *et al.* (1982) studied 11 rice cultivars differing in the tensile strength from 90 to 250 g for detachment of grains from panicle and found the shedding loss of grains ranged from 1 to 30% during harvesting. The average tensile strength of grains for zero field loss was estimated to be 174 g and a decrease in the average tensile strength by 10 g corresponded to an increase of 40 kg per hectare in field loss of grains. Jin *et al.* (1982) found that grain shedding in rice was controlled by a dominant gene for formation of the abscission layer at pedicel, a dominant gene for cracking of cells in the abscission layer, and other genes. Although genetics of grain shedding is not completely elucidated, non-shedding *indica* × *japonica* rice cultivars have been bred in Korea, implying that similar development of non-shedding *indica* cultivars is possible.

Registration and guidelines

Because gene flow is a slow and complicated process, preparation and evaluation of supportive data on long-term environmental impacts are not easy. When the UK Advisory Committee on Releases to the Environment (ACRE) was first faced with the assessment of GM OSR in 1994, the minority of the committee objected to commercialization because of the large uncertainty of the degree and consequences of gene flow; however, the majority opinion was accepted (Hill 1999). In the present system in most countries, data preparation for registration is the responsibility of GM seed company which may result in their overlooking of adverse long-term effects of gene flow on the ecosystem. More active information gathering from scientists may be required during the process of registration. To obtain more reliable data, sounder research on weed biology, especially reproduction biology, using standard experimental design systems and modeling studies of gene flow should be encouraged. Establishment of guidelines in the conditions for commercial release of a GM crop is very important. The guidelines include isolation distance, separate handling of GM seeds and products during transportation, storage and management, proper labeling, back-trace systems, education for seed sellers and farmers, herbicide application and system for hearing prompt feedback from scientists and farmers, and so on (Orson & Oldfield 1999). Mandatory monitoring at the onset of commercial release may be a good protective measure. Guidelines for handling GM cereal crops also should be established thoroughly because cereal can also serve as seeds. The seed company should play key roles in establishment and operation of the guidelines. A legally enforced system would make the whole system sounder. The risk of GM crops may vary in each country due to different environment and cultural practices.

Cultural practices

Champolivier *et al.* (1999), based on the results of an ongoing multi-year and multi-crop monitoring study, which began in 1995 in France, pointed out that long-term observations of the impact of GM crop cultivation under current agricultural practices should be performed in order to build suitable agronomic management and design a monitoring system, in addition to, and separate from, the evaluation carried out with the regulation process before marketing. They also suggested that a more integrated crop management than is presently practised should be required for GM crops to be cultivated. To manage the risk, rotation is recommended even though rotation is not always applied

easily in some systems. Rotation with after-crops, or yearly rotation with different crops, or even with GM crops with different traits, should be planned carefully and communication channels between adjacent farms should be established systematically. Monoculture causes many adverse effects on agriculture including dominance of certain diseases, insects and weeds. The composition of weed population and their relative competing ability can be altered by crop species and agricultural practice. Repeated cultivation of a specific GM crop over years may create a tremendous selection pressure to develop a weed resistant to the herbicide as the result of gene flow or mutation. This has been shown in monoculture with a single herbicide to evolve herbicide-resistant weeds (Gressel and Segal 1978; Powles *et al*. 1998). Herbicide rotation is important, too. Remaining herbicide-resistant crop seeds in the field can readily become volunteer weeds, increasing chance of leakage of the transgene, if the same herbicide is applied in the following seasons (Gressel 2000). Because a herbicide resistant trait usually does not reveal fitness advantages without the specific herbicide treatment, which is different to pest-resistant traits, herbicide rotation plays an important role in reducing the risk. Weed control of sexually compatible weeds in the vicinity, harvesting at the right time to reduce loss of seeds and other cultural practices such as cleaning equipment and facilities, field record keeping and monitoring should be implemented according to the guidelines.

CONCLUSION

Gene flow is an obviously complex but natural phenomenon with various factors involved. Introduction of a GM crop resistant to a specific herbicide creates a huge selection pressure to evolve herbicide resistance in weeds through gene flow and mutation. This evolutionary change is a very slow and nearly invisible process until it becomes irreversible and a reality. Because consequences of gene flow may only be detected when the impact is already beyond the manageable range, more cautious assessment and management may be the wise choice. The statement 'because any harm will be irreversible, the decision about acceptable harm should be a matter for wider social debate, particularly given considerable scientific uncertainty which affects the confidence that can be placed in any present day estimate' (GeneWatch 1998) may not be too conservative in the aspects of possible unexpected behavior of the transgenes, which have not evolved with the rest of the genome (Duke 1999). The estimate of potential gene flow may be greater than that of actual gene flow, and it takes time for some of potential gene flow to become actual gene flow. How much potential gene flow becomes actual is unknown and dependent on species and situation. Once the evolutionary change due to gene flow becomes detectable, it is generally irreversible. Thus, assessment of gene flow should include potential and actual gene flow using direct and indirect methods reviewed above. Additionally, the assessment must respect the identity of each country or region, as the risk can vary greatly from one agricultural system or practice and structure of wild flora to another. Even in the countries where GM crops are not cultivated, the assessment should be performed if the country imports bulk unprocessed GM crop commodities such as cereals or oilseed crops because there can be unintentional release into the environment during loading, transporting and distributing the goods. With such a comprehensive consideration and systematic approach employing various methods, accurate assessment of gene flow would be possible, and then it can provide a practical and useful basis for effective and appropriate management of gene flow from GM to non-GM crops.

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