SESSIONS 7 AND 8

GENE FLOW – THE FUTURE

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Concerns about gene flow and the implications for the development of monitoring protocols.

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ABSTRACT

The possibility that genes will move from crop plants to wild species, as well as to volunteers and to neighbouring non-GM crops, are high on the list of concerns about genetic modification. This paper gives an overview of the way the concerns have been expressed by some influential groups. It then discusses how they might be met by monitoring regimes, and what questions need to be addressed in order to construct such regimes.

INTRODUCTION

Concerns about gene flow are not new. The possibility of gene flow and its possible consequences were amongst the set of concerns about the potential environmental impacts of releases of genetically modified organisms that gave rise to European and UK regulations. The 1989 report from the Royal Commission on Environmental Pollution (RCEP 1989) which was influential in the development of the EU and UK regulatory systems, observed that "The extent to which genes, especially novel genes, may spread is an important uncertainty in assessing the risks in the release of genetically engineered organisms (GEOs)". The report recommended a system of scrutiny that would include examining the likelihood of gene transfer, and put considerable emphasis on the need to develop techniques for monitoring the spread of released GEOs and their introduced genes.

The UK went on to evolve a system of scrutiny, working to the requirements of EU Directive 90/220, that required those proposing to make a release to submit a risk assessment, including answering questions about potential gene flow. For the first 3-4 years of the system, many applications were very cautious in their nature, voluntarily proposing to limit gene flow by means such as preventing flowering of the crop, ensuring large isolation distances, and removing potentially sexually compatible wild species in the vicinity of the release. These measures were not necessarily linked to any identified hazard that could be realised as the result of gene flow - they presumed that gene flow was itself something to be avoided. This trend had three important consequences: it gave the regulatory system the appearance of being highly 'precautionary' in its nature; it simultaneously gave the impression that gene flow was a bad thing *per se*; and it yielded very little useful data about the potential for gene flow in the field.

Thus, when the EU and UK regulatory systems were faced in 1994 with the first application to commercialise a GM crop, the Plant Genetic System's (PGS) hybrid and herbicide-tolerant oilseed rape (PGS 1994) the issue of gene flow came more sharply into focus. The applicants took the view that gene flow to sexually compatible wild relatives of rape could not be ruled out, but that it would be at a low level, and because of the nature of the traits inserted in the rape, would not have deleterious consequences. A majority of the UK Advisory Committee on Releases to the Environment (ACRE) took the same view. A minority of the committee

objected to the commercialisation on the grounds that there was still a large measure of uncertainty about the extent of, and consequences of, gene flow (ACRE 1995). The UK Government accepted the majority advice.

To many outside the regulatory system, the UK decision on the PGS application marked a step change in the risk assessment of GMOs - a move from a precautionary approach to one where releases were to be allowed to go ahead with an unacceptable level of uncertainty. Since that point, concerns about gene flow have figured prominently in representations about GMOs. To illustrate this, some of those representations are outlined below.

A 1995 paper from the WorldWide Fund for Nature (WWF 1995) considered primarily what are often referred to as the 'indirect' effects of gene flow: "Transfer of herbicide resistance to weeds, even at low frequency, could clearly create enormous problems for weed control, and reduce the effectiveness of specific herbicides. In such situations, farmers would have fewer options in their weed control practices, and could be forced into using even more environmentally damaging chemicals. Weeds resistant to particular herbicides would have a selective advantage in systems where those herbicides are used".

A 1998 paper issued by Greenpeace International (Fromwald & Strauss 1998) was concerned about uncertainties. It stated: "Current scientific studies and knowledge demonstrate that GE oilseed rape, when commercially released in Europe, may transfer genes to other oilseed rape and wild related species. These hybrids and the GE oilseed rape itself may become a permanent feature of ecosystems and fields. Their overall effects are unpredictable, and once these species are introduced it may take tens or even hundreds of years to recognise their effects".

Like WWF, Greenpeace highlights the possible indirect effects on the use of chemicals: the spread of the tolerance gene to weedy relatives would cause the emergence of glufosinate resistant weeds, therefore creating even further problems of weed control for farmers. The GE oilseed rape and hybrids resulting from cross breeding between the GE oilseed rape and wild related species is also likely to spread onto organic farmers' fields, polluting organically grown products. This could force farmers out of environmentally sound farming methods since organic farming does not allow the use of GE crops, and consumers expect organic products to be free of genetically altered ingredients. Allowing GE oilseed to be grown on a large scale in Europe means undermining environmentally sound farming".

A 1997 briefing document from the Royal Society for the Protection of Birds (RSPB 1997) cites as one of the possible hazards of releasing GMOs into the environment: "The transfer of the introduced genes from the GMO to other species, which subsequently become established as 'pests'". RSPB also states that: "there are many potential effects on bird populations of GMOs which become established 'pests' within the environment including weedy crops or hybrids displacing other plants that are important food sources for birds". RSPB's concerns are more about the identifiable hazards of gene flow than about gene flow *per se*.

The GeneWatch report Genetically Engineered Oilseed Rape: Agricultural Saviour or New Form of Pollution? (GeneWatch 1998a) addresses gene flow as one of the foremost environmental risks associated with genetically engineered organisms (GEOs). The briefing document accompanying the report refers to gene flow as 'genetic pollution' (GeneWatch 1998b). The report notes that it is possible that GEOs will breed with native species, and will

transfer introduced genes, and states: "these foreign genes, which could not have entered the native gene pool through natural mechanisms, may become established and alter the genetic diversity of native flora and fauna". The report posits that "in the case of oilseed rape, gene flow to native flora in the UK is inevitable". The report acknowledges that hybridisation events are likely to be low frequency but states that: "the low frequency with which hybridisations take place in experiments cannot be equated with safety. As more varieties of GE oilseed rape are commercialised, the likelihood of harm will be increased"

The report asks the million-dollar question: "if gene introgression occurs - does it matter?". According to GeneWatch there are several causes for concern: "these include matters of immediate practical importance and more intangible concerns about altering genetic diversity so fundamentally and what this means for future generations". The main concerns discussed in the report are that alterations to the genetic makeup of plants could make them 'fitter' and create problem weeds for farmers or alter local ecosystems; and that gene introgression could reduce the fitness of native plants and lead to a reduction in numbers or local extinction of a species. The report points out that "the impacts of gene flow may not be recognised for many decades" and that "once gene introgression has taken place, it will be irreversible".

The GeneWatch report also reviews the advice given in 1994 by the Advisory Committee on Releases to the Environment (ACRE) on the PGS application and challenges two of the key elements of the advice: that gene flow to wild species will be minimal; and that gene flow is unimportant. On the extent of gene flow, the GeneWatch report draws attention to research on hybridisation published since the 1994 evaluation, indicating that hybridisation in the field may be more likely than originally supposed. On the importance of gene flow, the report notes that guidance on 'harm' which ACRE had been involved in developing with the Department of Environment, Transport and the Regions (DETR) "makes the assumption that genetic pollution *per se* is not harmful unless it is connected with a measurable change in species numbers or selected ecosystem parameters". GeneWatch challenges this assumption and states that "because any harm will be irreversible, the decision about acceptable harm should be a matter for wider social debate, particularly given the considerable scientific uncertainty which affects (the) confidence that can be placed in any present day estimate".

GeneWatch called on the Government to withdraw consent to market the PGS oilseed rape and undertake a re-evaluation of the environmental safety aspects, including a systematic examination of the impact of uncertainties on the risk assessment.

Friends of the Earth's 1997 Briefing Sheet on Genetically Engineered Oilseed Rape (FOE 1997) lists ten concerns about Glufosinate Tolerant Oilseed Rape, one of which is that "Glufosinate tolerance will spread to weeds". The briefing states that "Oilseed Rape can cross breed easily with other plants in the Brassica family such as wild turnip and wild radish. Research shows that these too can become herbicide resistant if crossed with the genetically engineered oilseed rape. Therefore the prospect of common weeds becoming agricultural pests (often labelled as 'superweeds') is very real and may lead to more toxic chemicals being used to control them." Friends of the Earth called for a moratorium on the growing of genetically engineered crops until the implications have been fully evaluated.

Friends of the Earth's 1998 court case against DETR attempted to establish that gene flow was possible between a GM maize crop and a nearby organic sweetcorn crop, and would result in

unacceptable 'pollution' of the organic crop. The case highlighted the fact that whatever the separation distances, gene flow could never be entirely ruled out or the extent of it accurately predicted, and that the crucial issue was the acceptability of any amount of gene flow.

English Nature's evidence to the House of Lords 1998/1999 enquiry (HOL 1999) had gene introgression at the top of a list of concerns. Uncertainty figures again: "these (introduced) genes have not been exposed to selective pressures in the habitat of the original organism, and may have unpredictable effects if they outcross into wild relatives". The report also states: "Outcrossing to native species is likely to be a rare event, but with increasing acreages of GM crops, will occur more often. We are concerned not only about the possibility of HT (herbicide tolerant) hybrids becoming persistent weeds, and insect resistant hybrids having adverse effects on farmland insect biodiversity, but also that outcrossing risks harm to biodiversity in other semi-natural habitats".

English Nature (EN) notes that: 'There are existing experimental methods of genetically modifying plants which significantly reduce the risk of gene transfer, but these are not being developed as a commercial priority". EN recommends that "if the regulatory process required that the risks of gene transfer were minimised during the developmental state of the product, it would stimulate further research and development of these safeguards in many crops".

Four main categories of concern emerge from these statements:

1. That gene flow constitutes unacceptable 'genetic pollution', whatever the consequences.

2. That gene flow will contaminate organic or other non-GM food crops;

3. That gene flow will occur and will have unpredictable consequences;

4. That gene flow will have identifiable deleterious consequences; such as spread of herbicide tolerance or insect tolerance in a way that has direct or indirect ecological impacts.

RESPONDING TO THE CONCERNS

The concept of 'genetic pollution' as a hazard in itself, independently of its consequences, tends to annoy scientists in the field. They point out that genes already move between species in nature, albeit rarely, and thus even in this GMOs do not present unique considerations. Some have argued that to try to avoid all 'genetic pollution' is to attempt to freeze the environment at a particular point in time and to deny the processes of evolution. Objections to the flow of genes as being 'unnatural' are often labelled as religious or quasi-religious, i.e. more a matter of doctrine than of science.

However, it is important to recognise that genetic manipulation does seem to represent a 'step too far' to some people, even if they can't articulate in strict scientific terms just what that step is (Grove-White et al 1997). It is possible to accept that we routinely intervene in nature, but simultaneously to object to particular interventions, or ones that lead to a change of scale - and the movement of genes between species will certainly increase in scale as a consequence of genetic manipulation and subsequent gene flow. The problem for policy-makers is knowing

how many people hold this kind of view, or how many would if they had enough information about the techniques and implications of GM crops. Detailed public consultation is needed to help ascertain this.

The issue of contamination of organic and other non-GM crops has received much more attention. Friends of the Earth and the Soil Association went to court in 1998 on behalf of an organic farmer who feared that the organic status of his crop would be jeopardised by cross-pollination from a GM fodder maize crop. Current organic standards state that "there is no place for GMOs in organic agriculture" which the Soil Association has interpreted as meaning that there should be no cross-pollination and thus contamination of organic crops at all. This would be very difficult to ensure even with large isolation distances, given the possibility that the pollen of some crops can travel for several kilometres, and even the standards for certified seed allow for some contamination.

There is as yet no resolution to this problem, and the case highlighted the fact that there is no mechanism for consultation between the growers of GM and the growers of non-GM crops, in case cross-pollination presents a problem. This may be an issue not just for organic growers, but for those undertaking to provide non-GM sources to retailers, some of whom want to offer own-brand products that are GM-free. It will also be an issue if crops are used as factories for non-food products such as pharmaceuticals and chemical feed-stocks, where it may be important that such substances do not enter the food chain through contamination of neighbouring crops. It would be unwise to put such substances into cross-pollinating crops in the first place, but in case that scenario does arise, there will need to be a regulatory mechanism for ensuring separation.

Unpredictability is also difficult to deal with in the regulatory regime. There are those that argue that avoiding all areas of unpredictability, by constantly invoking the precautionary principle, implies not getting out of bed in the morning. In order to reap benefits we must live with uncertainties, or there will be no progress. The counter-argument is that the regulatory system, and specifically the risk assessments that accompany applications for release consents, fail to acknowledge either the uncertainties or the possible benefits, and certainly do not offer a mechanism for weighing up the two. There is no way of deciding what constitutes an acceptable level of uncertainty in the context of a particular application. Scientific assessments tend to be strong on what is known rather than what is not known, and many critics would be satisfied simply by an honest statement of the gaps in our present knowledge.

DEVELOPING MONITORING PROTOCOLS - QUESTIONS

The fourth category of concerns, that gene flow will lead to direct or indirect damage to the environment of an identified kind, is more amenable to research and monitoring. However, some of the possible impacts are more easily monitored than others. The following are some of the questions that have to be answered before useful monitoring regimes can be constructed.

1. What **kinds** of gene flow do we want to monitor? Flow to wild relatives; to volunteers of the crops; to neighbouring crops? The factors monitored will depend on which of these, or perhaps all of them, are of concern.

2. It is also important to confirm whether the scale, perhaps better expressed as the **frequency** of gene flow is important in itself, or whether the focus should be on the **effects** of gene flow. If the latter, it is debatable how far assumptions about, and confirmation of, frequency is important, since it may be possible to study the effects of, for instance, hybridisation, without knowing how often it happens. On the other hand, there may be cases where the effects are related to the amount of gene flow happening, for instance where the concern is the rapid build-up of volunteer populations carrying introduced genes.

3. If wild relatives are the concern, one of the first considerations will be how much we know about **potential recipients** of introduced genes. The experience with oilseed rape has been that, as research has progressed, it has indicated that more wild relatives are capable of producing hybrids in the field than was thought when the early risk assessments were prepared (Gray & Raybould 1999). This is important because the distribution of those wild species thought to be candidates for hybridisation may determine which areas are targeted for monitoring.

4. What is the **distance** over which we want to monitor? This will be partly determined by what we know about how far pollen can travel, both from and to a crop, as well the issue above about the distribution of related wild species. It will also be strongly driven by the resources available to those monitoring.

5. What should be the size of the **sample** of wild plants to be sure of confirming assumptions about frequency of hybridisation (if frequency is felt to be important). This depends largely on what we think the hybridisation frequency might be, which in turn depends on having made the right assumptions about potential recipients (as in point 2 above). If the hybridisation frequency is assumed to be very low, the sample needs to be very large in order to detect any hybridisation, and confirming the frequency with any degree of accuracy will be difficult. If the hybridisation frequency is assumed to be high, the sample can be smaller. There is also emerging research (Bergelson 1998) that indicates that transgenes may intrinsically be more likely to transfer to near relatives than genes introduced by other means, which complicates assumptions about frequency, but which may also mean that there are more hybrids available for analysis.

6. Having decided the distance and area under scrutiny and the sampling methodology, we have to ask whether or not there are adequate **testing techniques**, and how practical these are to carry out on a large scale. A hybrid of a GM crop and a wild plant may not express the GM trait in a way that can be directly tested - for instance by becoming herbicide tolerant and therefore detectable by applying the herbicide. In such cases molecular analysis will be needed, which is time consuming if a large number of plants are to be tested.

7. In considering the **effects** of gene flow, one of the key concerns is whether introgressed genes will give the plant enhanced **fitness,** making it better able to compete in natural environments, and thus possibly decreasing the populations of other species. Here we need to decide what are the valid measurements of fitness, and over how many generations these need to be monitored to yield a meaningful result. A first generation hybrid may suffer a loss of fitness, which is then enhanced by back-crossing with the original wild-type.

8. In any assessment of effects it will be important to have **base-line data** about species diversity and levels of populations before there is any gene flow. Otherwise it will be impossible to assess how species composition has been altered.

9. Another key concern is whether flow of introduced genes such as those for insect resistance, if they become incorporated into wild species outside the agricultural environment, could affect **insect diversity**, and whether this would be felt higher up the food chain, for instance by affecting food supplies for birds. Here base-line data will be particularly important, not only about populations of plants that could be recipients of the genes and about the insects associated with them, but also about non-target effects, i.e. toxicity to insects that prey on the insects that eat the plants (Hilbeck et al 1998). Information would also be needed about food-webs, i.e. what species are dependent on particular insect species, and how much effect on insect populations would have a knock-on effect on those species.

10. For flow to **volunteers,** it needs to be considered why this is of concern - because volunteer populations will present weed problems in themselves if they take on introduced genes (for instance by becoming herbicide tolerant and therefore not amenable to eradication with that herbicide); that volunteer populations could become a reservoir where a variety of different introduced genes could become 'stacked', producing volunteers with multiple tolerance; or whether the reservoir of introduced genes in volunteer populations could provide a concentrated source of gene flow to wild relatives. The most relevant questions are likely to be those about **distance** to monitor, appropriate **sample size,** and the availability of appropriate **testing techniques.**

11. For flow to **neighbouring crops**, all the questions relevant to volunteers will be important, but an additional consideration will be how much consultation there will need to be with neighbouring farmers to ensure access to the crop to be monitored.

12. For any monitoring regime, a key issue is the **time** over which it is conducted. Effects in the field such as enhanced fitness may take many generations to show up, so it is worth seriously considering whether a three- or even a five-year monitoring programme will yield meaningful results. Monitoring programmes that conclude that nothing is happening will be criticised as lacking credibility and wasting money if they are constructed in timeframes that could not reasonably be expected to detect any effects.

All these questions need to be debated among a wide range of interest groups, not just among the scientifically qualified, before monitoring regimes can be constructed that will satisfy the concerns about gene flow.

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Molecular aspects of multiple transgenes and gene flow to crops and wild relatives

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ABSTRACT

The consequences of transgene stacking need to be assessed to provide information to regulatory bodies concerned with genetically modified crops (GMOs). How transgenes will interact depends on the degree of homology between their gene sequences or with resident genes, the copy number present, and their expression. Transgene stacking provides opportunities for plant breeding, by combining traits unobtainable by conventional methods. However, such changes could be transferred to wild populations and impact on the environment. Multiple transgene flow to crop relatives needs careful consideration during biosafety assessment to prevent undesirable combinations becoming established in wild plant species. The design of transgene constructs could play some part in the reduction of environmental impact by targeting gene products more effectively, using inducible promoters and reducing current reliance on constitutive types.

INTRODUCTION

Plant transformation technology has become an established method to introduce new traits into crop plants. This technology is continually being applied to more plant species, with the intention to improve them for human use. The technology is advancing and new ways are being developed to transfer more complex traits and larger DNA fragments to plants, for both research and commercial uses. The first crops developed from transformation technology have already reached the global market place. So far these crops only contain a single construct but, through either deliberate breeding or gene flow, constructs could become stacked together in the same crop plant or in related species. The presence of such multiple transgenes, whether by design or accident, is an important aspect of biosafety assessment. Molecular aspects of gene stacking need to be highlighted to aid evaluations of transgenic crops before they are released into the environment. This paper is intended to address some of the salient molecular issues of multiple gene constructs both in transgenic crops and via gene flow to related crops or wild relatives.

PRESENCE OF MULTIPLE TRANSGENES IN CROP PLANTS.

Plant breeders will want to combine different constructs together to obtain phenotype combinations unavailable by other means. Multiple transgenes will frequently be introduced by sexual hybridisation between single trait elite breeding lines, followed by extensive progeny screening. Table 1 illustrates the possible combinations of traits that may be achieved through breeding. Some of these combinations are more desirable than others, and some have already been produced e.g. herbicide tolerance and male sterility. Combining two or more herbicide tolerance transgenes together is not considered to be an attractive idea, although there are proponents of this approach. Transfer of two herbicide tolerance traits to relatives would make it more difficult to control volunteers or weeds containing these genes. Other combinations are less likely to be disadvantageous if they were to be combined in weeds e.g. male sterility with oil modifications.

Multiple transgenes could also occur through gene flow between sexually compatible transgenic crops planted close together. This is likely to occur at a higher frequency than transfer to wild relatives. Initially at least the probability of the production of multiple transgenes in weedy species is likely to be very low. Availability of related species, along with survival, fitness and fertility of their hybrids will define how quickly transgenes will transfer in the short term, however, in the long term it is likely that multiple transgenes will be found in weedy populations.

STABILITY OF TRANSGENE EXPRESSION

Stable transgene expression is a prerequisite for a commercially viable crop. Usually many independent transformants are assessed in order to find individuals with both appropriate expression levels and transgene stability. Such work has helped to confirm classical genetic evidence of different allelic and non-allelic gene interactions known to affect gene expression (Nap *et al.* 1996). Most of these interactions result in down regulation of gene expression. Gene silencing of transgenes has been a perplexing problem for both researchers and companies alike. Extensive research has shown that transgene silencing is caused by a variety of different mechanisms: position effect, transcriptional and post-transcriptional silencing mechanisms.

Position effect

Analysis of a population of primary transformants reveals a wide range in transgene expression levels, which is often attributed to the 'position effect' - the site of integration of the transgene into the plants genome. These differences occur because of the random way transgenes are integrated into the genome. Transgenes integrating into hypermethylated regions of chromosomes can become methylated and this can inactivate their transcription (Meyer 1995). Introducing matrix attachment region sequences (MARs) along with the desired transgene can moderate expression level variation, due to the site of integration (Mlynarova *et al.* 1994), but have not prevented gene silencing events (Vaucheret *et al.* 1998).

Transcriptional gene silencing

Matzke *et al.* (1989) first observed transcriptional silencing of transgenes, when they sequentially transformed tobacco with two different constructs. These constructs had some sequence homology between each other at the promoter level. Some of the resultant progeny were unexpectedly sensitive to the antibiotic used for selection. Further work showed that this effect could also be observed when two transgene loci were brought together by sexual hybridisation. Analysis of the loci involved in this silencing effect showed multiple copies of one of the transgenes, were present at the locus which could silence any incoming, homologous transgenes (see Matzke *et al.* 1996 for a review).

Post-transcriptional gene silencing

Post-transcriptional gene silencing was first observed with chalcone synthase (*chs*) and dihydroflavonol-4-reductase transgenes when they were introduced into petunia. Instead of over-production of the gene product, suppression of both transgene and endogenous genes occurred. Previously it was known that reversing the coding sequence (antisense) could silence genes, whereas genes introduced in a sense orientation would be over-expressed. It is now established

that genes in the sense orientation can also silence expression. This type of gene silencing has been seen in many species and has now become an alternative method to silence plant genes (see Depicker & van Montagu for a review).

Over the last decade considerable research efforts have probed the mechanisms of plant gene silencing (reviewed in Kumpatla *et al.* 1998; van den Boogaart *et al.* 1998). Similar silencing systems are also known in fungi and it is postulated that all genomes have evolved similar defence mechanisms (Kumpatla *et al.* 1998). Gene silencing mechanisms are not fully understood, but it is thought that many different levels of interaction are involved in the recognition of invasive sequences. Recent evidence has suggested that a signalling system is operational, which spreads the silencing command throughout the plant (Voinnet & Baulcombe, 1997). Further evidence to support this signalling theory came from grafting experiments by Palauqui *et al.* (1997), who showed that the silencing phenotype was graft transmissible.

INTERACTIONS OF TRANSGENES WITH THE ENVIRONMENT

Pathogen interaction

Evidence for viral interaction with transgene constructs is well known (Baulcombe & English 1996) and recent data has shown gene silencing can also be initiated by viral infection of transgenic *Brassica napus*. When infected with cauliflower mosaic virus (CaMV), a gradual decline in transgene expression was observed in plants containing transgenes driven by the viral CaMV 35S promoter (Al Kaff *et al.* 1998). Transgenes driven by other non-homologous promoters remained active in infected plants. This indicated the silencing mechanism relied on sequence homology between the virus and transgene to silence gene expression of both.

Heat stress

Transgene silencing can also be induced by changes in temperature. Exposure to both heat and bright light caused *chs* expression to become gradually weaker when petunia plants were grown under field conditions (Meyer *et al.* 1992). Walter *et al.* (1992) found 95% of cells were silenced after suspension cultures of glufosinate ammonium tolerant *Medicago sativa* were given heat shock treatment (37°C for 10 days). Heat induced transgene inactivation in whole plants was investigated by Broer (1996) using *Nicotiana tabacum*. A reduction of expression was detectable at 35°C, while all tested plants showed damage if exposed to 40°C for 10 days, decreasing the temperature back to 25°C restored the transgene phenotype. Further work by Neumann *et al.* (1997) showed temperature sensitivity or tolerance of transgenes was heritable. The phenomena did not correlate with copy number or to the allelic state.

Transfer and cultivation

Brandle *et al.* (1995) found that seeds sown directly into the field retained their transgene expression, but those transplanted from the glasshouse into the field suffered silencing. Other silencing events have been found to depend on environmental conditions (van der Krol *et al.* 1990; Hart *et al.* 1992; de Carvalho Niebel *et al.* 1995) while others are developmentally controlled (de Carvalho *et al.* 1992; Dorlhac de Borne *et al.* 1994).

COMBINING TRANSGENES

Plant breeders will want to combine different transgenic elite breeding lines (e.g. herbicide tolerance with quality traits) to produce new transgenic cultivars. This process is similar to conventional plant breeding where ranks of characters are combined by hybridisation, and selection sorts out the superior combinations. Stacking transgenes could potentially result in transgene silencing if the constructs have high levels of sequence homology either between themselves or with endogenous genes. Silencing can also occur where single copies of highly expressed transgenes are present (Elmayan & Vaucheret 1996). However, silencing is more likely where multiple constructs are located at a single locus. Strong silencing loci have been found which completely silence any incoming construct carrying homology to the first. Separation of the two in the next generation can still result in lower expression than control plants as the imprinting of the loci is retained for several generations (see Matzke *et al.* 1996) Such effects in breeding lines would result in the combination being discarded. High performing lines that survive the rigorous testing and selection procedure are likely to become commercial cultivars.

Accidental stacking

Little data is available on the effects of single transgenes in wild populations and even less for multiple transfers. Examining the literature for single combinations shows that transgenes will remain active in new hybrids and that they can be passed on through the generations (Chevre *et al.* 1998). This should also be the case for multiple transgenes, with the expectation that different constructs will segregate in a Mendelian manner in most instances. Only transgenes that are closely linked will remain together over the following generations. It is expected that progeny from crop x wild species hybrids may have a range of phenotypes for the introduced genes. Some gene silencing could occur, but in most instances we would expect expression. After the transfer of transgenes to wild relatives, they will be under the control of a new genetic background. This could influence transgene expression and the environmental impact it has. Dormancy characteristics of the crop x weed hybrid will also influence how long seeds containing transgenes can survive in the seed bank.

CONTROLLING TRANSGENE ESCAPE

In many instances the risk assessment will conclude that gene movement from transgenic varieties is no more hazardous than from conventionally bred varieties. In other cases, such as the production of specialist products (pharmaceutical substances and special oils) it may be necessary to control transgene movement. Various methods are emerging to control transgene escape from the crop to related plants. Some of these methods are more appealing than others, but all could influence transgene spread.

Transformation of plastids

This is a developing field where plastids are targeted for transformation instead of the nuclear genomic DNA (Dix & Kavanagh 1995). Transformation of plastids has been achieved for several model species, notably tobacco (Daniell *et al.* 1998) and *Arabidopsis thaliana* (Sikdar *et al.* 1998). As plastids have their own DNA which is mostly maternally inherited, any integrated transgenes are very unlikely to be transferred into relatives via pollen, although this does not preclude the

reverse hybridisation from occurring (e.g. the crop pollinated by the weed species).

F1 hybrids, male sterility

Many workers have been engaged in obtaining male sterility in crops. This has been achieved by both conventional and transgenic approaches. The advantages of such systems lie in the ability to produce F1 hybrids, which can be more productive than normal cultivars. Hybrids offer productivity advantages to farms but prevent farmers from saving their own seeds. The rigors of hybrid seed production will make it less likely that seeds for sowing will become genetically contaminated. Male sterility has also been considered as an isolating mechanism to reduce gene flow e.g. forest trees.

'Killer' constructs

Mechanisms by which fertile seed is prevented from being produced are being evaluated. These could potentially halt the spread of transgenic feral plants. Introgression of such traits into wild relatives of crops could influence ability to reproduce, but there is likely to be intense selection against individual hybrids expressing this transgene.

Construct design

The way a construct is designed could influence the spread or activity of transgenes. Consideration of the type of promoter to be used, and in what context, should become an integral part of construct design. It is clear from the work of Al Kaff *et al.* (1998), that the use of pathogen derived sequences in their natural plant hosts can result in transgene silencing. The use of tissue specific promoters rather than constitutive promoters may reduce some of the potential impact of gene transfer. For example, modified oil composition can be precisely targeted to seeds or particular parts of the seed, with all other plant parts retaining a 'wild type' phenotype.

Inducible promoters have now been isolated (Gatz & Lenk 1998), and could provide an efficient way to control transgene expression in field crops. For example, crops containing herbicide tolerance transgene driven by such promoters could be sprayed with the inducer, followed by the herbicide. This would control both unrelated weeds and non-transgenic weeds. Following harvest, a second round of spraying could occur with the herbicide only. Any crop volunteers or transformed weeds will be destroyed along with their non-transgenic counterparts as the transgene would be silent.

DISCUSSION

The introgression of multiple transgenes into related wild populations will occur for some of our transgenic crop plants. The genes that are transferred will determine the significance of such events. It should be remembered that plant breeding has already produced many novel combinations of genes for e.g. disease resistance, insect resistance and tolerance to environmental stresses, all of which have had the opportunity to be transferred to wild relatives. Effects of transgenes in wild populations are likely to be the same or similar to those seen for conventional plant breeding traits. If they convey selective advantage or are neutral in their effect, it is possible that they will be assimilated into the wild species genotype. Observations on the effects of some

transgenes have been shown to result in poor survival (Sweet *et al.* 1997), while other traits (Bt gene) have given greater advantage in an ecological niche and therefore could become established in the wild population (Stewart *et al.* 1997). Factors which could reduce the impact of gene stacking include; construct design, use of targeted constructs, less reliance on constitutive expression of traits and greater use of inducible promoters. These changes should help to reduce the rate of introgression resulting from selection and subsequent expression of multiple transgenes in the environment.

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Table 1. Deliberate stacking of transgenes in plant varieties

Characters*	Viral	Fungal	Bacterial	Insect	Breeding System	Stress	Morphology	Propagation	Herbicide	Pharmaceuticals	Protein	Starch	Oil
Viral resistance	✓✓	-	-	-	-	-	-	-	-	-	-	-	-
Fungal resistance	✓✓	✓	-	-	-	-	-	-	-	-	-	-	-
Bacterial resistance	✓	✓	✓	-	-	-	-	-	-	-	-	-	-
Insect resistance	✓✓	✓	✓	✓	-	-	-	-	-	-	-	-	-
Breeding system	✓	✓	✓	✓	✓	-	-	-	-	-	-	-	-
Stress tolerance	✓	✓	✓	✓	✓	✓	-	-	-	-	-	-	-
Morphology	✓	✓	✓	✓	✓	✓	✓	-	-	-	-	-	-
Propagation	✓	✓	✓	✓	✓	✓	✓	✓	-	-	-	-	-
Herbicide tolerance	✓	✓	✓	✓	✓✓	✓	✓	✓	✓**	-	-	-	-
Pharmaceuticals	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	-	-	-
Protein	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	✓	-	-
Starch	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	✓	-
Oil	✓	✓	✓	✓	✓	✓	✓	✓	✓	X	X	X	✓

✓ = Stacking probable; ✓✓ = Stacking already practised; ✓** = Stacking not recommended; X = Stacking unlikely

* Examples: Viral resistance - coat protein, replicase; Fungal resistance - chitinase, glucanase, lysozyme; Bacterial resistance - cecropin; Insects resistance - Bt, trypsin inhibitor, lectin; Breeding System - male sterility, self compatibility, self incompatibility; Stress tolerance - drought, cold, heat, salt; Morphology - flowering, dwarf, root form, pod shattering, canopy structure; Propagation - seed number, seed dormancy, vegetative propagation; Herbicide tolerance - glufosinate, glyphosate, bromoxynil; Pharmaceuticals - hirudin, anticancer, antibodies, vaccines; Protein - storage proteins; Starch - quantity and structure; Oil - quantity and type.