GM SCIENCE REVIEW

FIRST REPORT

An open review of the science relevant to GM crops and food based on the interests and concerns of the public

PREPARED BY THE GM SCIENCE REVIEW PANEL (JULY 2003)

Chapter 7

GENE FLOW, DETECTION AND IMPACT OF GM CROPS

7.1 INTRODUCTION

This Chapter of the Science Review considers the state of our knowledge on issues related to the transfer of genes (*gene flow*) from GM crops to plants and other organisms, the environmental impacts that might arise, methods for gene detection and means of controlling gene flow.

Whether gene flow matters will depend on its consequences (whether from GM or non-GM crops). However, if GM crops are to be grown on a commercial scale in the UK, gene flow will be an important factor in determining the terms on which non-GM, organic and GM agricultures might co-exist.

The dispersal of plant material does not necessarily constitute gene flow *per se*. Gene flow results in the combination of genetic material from individuals with different genetic backgrounds – for instance between different plant varieties or populations. A critical factor is whether these genetic combinations will persist in individuals in future generations and if they do, what significance if any, they might have. Gene flow from crops has implications for the maintenance of genetic integrity, an issue that has been raised with respect to gene flow from GM crops to semi-natural plant populations in this review.

Genes are transferred between sexually compatible plant species when they hybridise (cross) with each other - this is facilitated by pollen dispersal and cross-pollination. Genes can also be moved in seed and sometimes by other plant material that is capable of giving rise to new plants (e.g. potato tubers). In the UK, some crops can exchange genes with certain agricultural weeds or with plants living in semi-natural environments with which they share a close genetic relationship. Whether we have the knowledge to predict the extent to which transgene flow from crop plants to related species and genera could occur and the potential consequences for agriculture and the wider environments (e.g. increased invasiveness) is addressed in this Chapter.

Pollen is released in enormous loads into the atmosphere, and can travel over very great distances - therefore plants will be dusted with pollen from a diversity of sources. However, the vast majority of this pollen will not result in successful cross-hybridisation for any number of reasons e.g. sexual incompatibility, it does not land on the female parts of the plant, it cannot successfully compete with other pollen grains or because it is unviable. This Chapter does not deal with the presence of pollen (that does not pollinate the plant that it lands on) or dust from GM crops on non-GM plants as this does not constitute gene flow - although for some people it does constitute a form of GM ‘contamination’. Whether such GM plant material is likely to be more toxic and/or have greater allergenic potential than non-GM plant material is considered on a case by case basis in risk assessments. The science behind these questions is discussed in Chapter 5. In addition, sections 5.4 and 7.4 of this Review consider the potential for genes from GM crop material to transfer to microbes in the gastro-intestinal tracts of the humans or animals that consume it and to microbes in the soil.

A possible consequence of gene flow between different GM (and non-GM) varieties is 'gene stacking' (the accumulation of genes encoding different traits resulting from cross-pollination between different varieties of the same crop). This has been well documented in oilseed rape in Canada. The effect that stacked traits such as herbicide tolerance, but also in future a wide range of other traits, might have is an issue that has been raised in this Review.

Spilt seed or vegetative tissue (e.g. tubers) remaining after a GM crop has been harvested may act as a reservoir for transgenes. As GM volunteers (plants growing adventitiously from this residual seed or from vegetative material) can grow several years after the original GM crop is harvested they could mediate transgene transfer over time. GM material may also become mixed with non-GM and other GM varieties after crops have been harvested - in storage or further down the production and transport chain. This raises the issue of detecting unintended GM presence, which is also addressed in this Chapter.

The transfer of genes between plants by cross-pollination is sometimes referred to as vertical gene transfer (VGT). This contrasts with horizontal gene transfer (HGT), which refers to the non-sexual, non-parental-to-offspring processes by which genetic material can sometimes transfer between organisms with distant genetic relationships. In this Review the potential for gene transfer from GM crops to soil microbes and to viruses has been raised. There is concern from some quarters that this could lead to adverse effects on ecosystems and to the generation of new viruses. However, this supposes that gene transfer between plants and microbes/ viruses, actually occurs - the evidence will be discussed in this Chapter. The transfer of transgenes to gut microflora is not addressed here, this topic is covered in section 5.4.

Public concerns about GM were reflected in a report, produced as a result of a series of foundation discussion workshops under the GM Public Debate strand of the GM dialogue (see Chapter 2 - methodology). The questions of particular relevance to this Chapter are:

Could harm be caused by cross-contamination?
What will the effect be on 'natural' (non-GM) crops / wildlife?
What are the real experience of US farmers and consumers?
What controls and regulations/ legislation are in place?
What legacy are we leaving future generations?

More specifically, issues on gene flow, detection and impact of GM crops were raised under the Review at various open meetings, in contributions to the Science Review website and by GM Science Review Panel members at their meetings.

We consider four types of gene flow and the potential consequences of it occurring in this Chapter. Text in italics aims to encapsulate many of the public issues and concerns that have been raised on gene flow, detection and impact of GM crops during this review:

7.2 Gene flow between crop varieties

Can the extent and consequences of gene flow from GM crops to other crop varieties (GM and non-GM) be predicted and controlled? Is co-existence between GM and non-GM crops possible and can we detect unintended GM presence?

7.3 Gene flow from GM crops to agricultural weeds and wild relatives.

Can the extent and consequences of gene flow from GM crops to agricultural weeds and wild relatives be predicted and controlled? Could gene flow from GM crops generate superweeds or eliminate wild plant populations?

7.4 Can genetic material in GM plants transfer to soil microbes?

In nature, how important and prevalent is horizontal gene transfer between plants and microbes in the soil, and does the presence of transgenic DNA make this more likely to occur? To what extent are the ecological effects of horizontal gene transfer from plants to soil microbes predictable?

7.5 Can genetic material in GM plants transfer to viruses?

Can plant-virus-derived transgenes recombine with, and be transferred to viruses? If horizontal gene transfer is possible between GM plants and viruses could this result in new viruses that could cause irrecoverable damage to the ecosystem or to crops?

7.2 GENE FLOW BETWEEN CROP VARIETIES

Can the extent and consequences of gene flow from GM crops to other crop varieties (GM and non-GM) be predicted and controlled? Is co-existence between GM and non-GM crops possible and can we detect unintended GM presence?

7.2.1 Summary

Transgenes and native plant genes are dispersed in pollen and seed. For the most part gene flow takes place within a few metres of the plant, but it can occur over several kilometres. Seed is typically moved over much greater distances than pollen, both by natural seed dispersal and intentionally and unintentionally by humans. In addition, once seed is dispersed, it is much more likely to result in the establishment of a plant containing the genes in question.

Pollen-mediated gene flow and the separation distances employed to minimise it typically generates more public interest than the movement of genes in seed and this has been the case in this Review. However, the implementation of agronomic practices that minimise the dispersal of genes through seed is essential for maintaining gene flow below set thresholds, for example by limiting gene flow through volunteers, preventing unintended mixing of different seed lots and reducing the transportation of seed on agricultural machinery. However, the complete genetic isolation of most crops grown on a commercial scale, either GM or non-GM is not practical, at least in the foreseeable future.

Distance from a pollen source and cross-pollination frequency with neighbouring crops can be predicted for most major crops. Separation distances based on these predictions and agricultural practices that minimise seed-mediated gene flow have been employed to successfully minimise gene flow between non-GM crop varieties for economic (e.g. certified seed crops and identity preservation schemes for different types of maize) and safety reasons (i.e. separation of non-GM oilseed rape varieties containing high or low erucic acid levels). However, restricting gene flow between non-GM and GM varieties will potentially be on a much larger scale than anything that has preceded it.

The amount and potential consequences of gene flow are considered on a case by case basis for GM crop varieties and this forms part of a risk assessment on which the decision on whether to issue consent for release into the environment is made (please refer to Chapter 3).

The levels at which gene flow can be maintained for different crop varieties are significant in determining whether co-existence of different types of agriculture is feasible. There is little available evidence on how the different factors (seed purity, cross-pollination, the contribution of volunteers and the effects of seed mixing) affecting co-existence will combine if GM crops are grown on a commercial scale in the UK, this makes prediction difficult. Political decisions may ultimately affect whether co-existence is practical, in particular what thresholds are set for maximum GM presence in non-GM crops (and their products), whether conventional or organic. For some crops, maintaining thresholds of gene flow may be relatively straightforward, by employing separation distances and, more importantly, by reducing gene flow through seed. However, in other cases it may be difficult, and perhaps impossible.

In order to enforce maximum threshold levels of transgenic DNA in non-GM crops, the tools for accurately sampling, detecting, quantifying and identifying unintended transgenic DNA presence must be in place. Absolute thresholds for detecting specific fragments of DNA depend on the crop. As there are limits of detection, even though these are extremely low, zero transgenic DNA presence cannot be guaranteed using these methods.

Detection and identification of GM presence may be limited if genetic markers which identify the GM crop aren't available (e.g. in the case of unapproved GMOs). Genetic elements that are commonly used in GM technology may show that transgenic DNA is present but these will not identify its source.

'Gene stacking' (accumulation of genes conferring different traits as a result of hybridisation between different varieties) is not unique to GM crops. However, 'transgene stacking' could result in the combination of genes that would not be brought together in non-GM crops and were not intended to be brought together in approved GM crop varieties (although the potential and consequences of these traits combining would have been addressed in risk assessments). The potential consequences of transgene stacking is already a consideration in the release of new GM crop varieties in the UK but this is likely to become more complex if a range of different GM crop varieties are grown on a commercial scale.

The advent of GM crops that produce novel products such as pharmaceuticals, bioplastics or biofuels pose a new problem for regulators. However, this is not unique to GM agriculture, some oilseed rape varieties produce oil that is toxic to humans so must be separated from varieties that produce oil for food products. The existing practice of assessing each new GMO on a case by case basis is appropriate for regulating these new types of GM crops.

7.2.2 Introduction

Gene flow from GM crops to other crop varieties was discussed at open meetings held at the Royal Society of Edinburgh[1], the Royal Society[2] and the Institute of Grassland and Environmental Research in Aberystwyth[3].

Gene flow between neighbouring crop varieties has been taking place almost since agriculture began. Consequently, it has been important for growers to maintain agronomic characters specific to certain varieties and for seed producers to maintain purity standards for certified crop varieties. However, the advent of GM crops has significantly increased public and scientific interest in gene flow.

Genes are transferred between sexually compatible plants through pollen. Typically, there is an rapid decline in the amount of pollination that occurs as distance from a plant increases, however this decline becomes much less pronounced as the probability of pollination nears zero (Champolivier *et al*., 1999). Therefore, the vast majority of pollination will occur within a few metres of a plant, but there may be rare occurrences of cross-pollination at distances of a kilometre or more (Rieger *et al.* 2002).

[1] http://www.gmsciencedebate.org.uk/meetings/pdf/270103-transcript.pdf
[2] http://www.gmsciencedebate.org.uk/meetings/pdf/110203-transcript.pdf
[3] http://www.gmsciencedebate.org.uk/meetings/pdf/170303-transcript.pdf

The distance that pollen travels depends on a number of factors, for example: the type of pollen (i.e. how much is produced and how heavy the grains are) and the mechanism by which it is dispersed (wind/ insects or a mixture of both). If insects are involved, their behaviour will affect pollen dispersal. Climatic conditions will significantly affect dispersal (i.e. temperature, humidity, light, wind and rain) as will natural barriers such as surrounding vegetation and topography.

Although pollen dispersal provides a guide to the actual distances of gene flow there are a number of other important factors that must be taken into consideration. These include:

- The breeding characteristics of different crop varieties. For instance, some crops predominately self-pollinate (e.g. wheat and barley) whereas others have a higher degree of out-crossing (e.g. maize).
- Cross-pollination can only occur between plants that flower at the same time.
- Different types of pollen vary markedly in their ability to remain viable under different conditions (such as temperature and humidity). Therefore, the pollen may travel a long distance but it will not necessarily have the capacity to cross-pollinate.
- Competition between pollen from different sources (e.g. large amounts of local pollen versus small numbers of pollen grains arriving over long distances).

The potential for gene transfer is very different depending on the crop and the variety. For example, Hucl (1996) examined variability in the amount of self-pollination (in-breeding) of 10 wheat cultivars and found rates varied between 97.7% (cv. CDC Makwa) and 93.95% (cv. Oslo). Maize on the other hand is more likely to cross-pollinate (under normal field conditions at least 95% of the ovules are fertilised by pollen from other plants, Poehlman, 1959). Many plant varieties have self-incompatibility mechanisms that restrict self-pollination (Kao and Mc. Cubbin, 1996; Takayama and Isogai, 2003), however varieties grown for grain or seed often have high degrees of self-pollination.

The relationship between distance from pollen source and the cross-pollination of neighbouring crops can be predicted. In a report for the then Ministry of Agriculture, Fisheries and Food, Ingram (2000) identified robust, representative data sets and applied them to typical farm situations. The report proposed recommended separation distances to restrict cross-pollination frequencies to below 1%, 0.5% and 0.1% for non-seed crops of sugar beet, maize and oilseed rape. These recommendations form part of the basis for current assessments of gene flow.

The movement of genes in seed may be a more significant factor than cross pollination in contributing to gene flow but this hasn't, so far, received the public interest that separation distances have, possibly because most GM field trials, including the large-scale farm-scale evaluations, have been managed to minimise the possibility of viable seed being set. Agricultural practices that minimise the movement of seed are very important in maintaining levels of genetic purity.

As with pollen, seeds can transport genes away from their source, however unlike pollen, seeds can also mediate gene flow over time. Depending on the type of seed, it may lie dormant in the soil for many years before germinating. Plants that grow from seed, or from vegetative structures (e.g. beet tops or roots) left by a previous harvest are referred to as volunteers (Downey 1999). Volunteers and plants growing from stubble are a potential source of transgenes to crops in future harvests.

The unintended presence of GM seed in non-GM seed lots may also facilitate the transfer of transgenes into conventional crop varieties. This adventitious GM presence may be the result of hybridisation events between different varieties, or the accidental post-harvest mixing of seed. The practice of saving a proportion of seed from one harvest to sow in subsequent years (so-called 'farm-saved seed'), rather than buying seed produced commercially, is likely to increase the likelihood of seed mixing – particularly on farms growing GM and non-GM varieties of the same crop.

A harmonised EU system for tracing and labelling GMOs and products derived from them at all stages of their placing on the market is being developed. From a technical standpoint it is important that methodologies are in place that can accurately and reproducibly detect unintended GM presence at levels dictated by the legislation. Annex V describes the proposal for new legislation concerning the traceability and labelling of food and feed derived from GMOs.

The thresholds proposed for the unintended presence of approved GMOs in conventional crop seed are between 0.3 and 0.7% depending on the crop (Commission proposals on thresholds for the adventitious presence of approved GMOs in seeds, document: SANCO/1542/02July2002)[4]. These were calculated to broadly support the food labelling threshold. It is important to note that the thresholds set for GM presence are pragmatic as their role is to give consumer choice – this contrasts with other 'contaminants' which may have safety implications above set thresholds.

Ultimately, for this legislation to be effective there must be internationally approved monitoring, sampling and detection methods for all crops (and products derived from them) that are capable of facilitating the detection and quantification of GM presence at, or below any threshold levels that are set. Although it is outside of the remit of this review paper, it is important to emphasise that technical ability is not the sole consideration in determining whether legislation can be enforced – there are many political considerations such as economics and liability.

7.2.3 Range of views and quality of evidence

To what extent does crop to crop gene flow occur and how predictable is it?

This section is principally concerned with pollen-mediated gene flow because of the interest expressed in this issue during the GM Science Review. However, it is a widely held view of experts in this area that the implementation of agricultural practices that limit the movement of seed is critical in minimising gene flow from GM varieties.

A point that is often made is that once genes are transferred out of GM crops they cannot be 'recaptured', or once the genie is out of the bottle it cannot be put back. The next section in this Chapter (7.3) considers the potential for transgenes to persist if transferred from GM crops to sexually compatible weedy and semi-natural plant populations.

There are polarised viewpoints on gene flow from GM crops. One group considers that any amount of gene flow is unacceptable as they do not want the food they eat to be derived from

[4] http://www.defra.gov.uk/corporate/consult/approvedgmos/sanco1542.pdf

GM crops, whether it contains transgenic DNA or not (highly processed products such as sugar and oil do not contain DNA or protein). Conversely, others consider that gene flow is only a problem in particular instances, for example if there is gene flow between high erucic acid varieties of non-GM oilseed rape and low erucic acid varieties used in food products (Bilsborrow *et al.* 1998). Since the risk is specific to the varieties involved and not a generic problem of gene flow *per se*, and the amount of gene flow differs in different crops under different circumstances, the case by case assessment of each crop/trait combination is appropriate.

There is a large body of evidence on gene flow over relatively short distances (i.e. hundreds of metres rather than several kilometres), for major crop plants. This comes from:

(i) Practical experience in limiting gene flow between non-GM varieties

Certified seed schemes (require seed production above set levels of genetic purity). However, the amount of detailed information from this source is generally limited e.g. minimum separation distances are adhered to but the actual separation distances used are not recorded. In addition, it is possible that in some cases, the presence of unwanted genotypes (so called 'off-types') have been under-estimated since they are often screened on the basis of visual characteristics.

Non-GM field crops separated to maintain product purity. There are limited examples where measures have been taken to restrict pollen-mediated gene flow between different non-GM varieties of the same crop. These have been employed in protecting the characteristics of sweetcorn from other types of maize and in preventing contamination of low erucic acid varieties of oilseed rape (the oil is used in food products) with pollen from high erucic acid varieties as they produce oil that is toxic.

(ii) Scientific studies

There is a large body of evidence from scientific experiments on pollen-mediated gene flow and this has increased in recent years due to the interest in GM crops (reviewed by Treu and Emberlin, 2000[5]; Ingram 2000; Eastham and Sweet, 2002). There are different views on whether separation distances based on these data are adequate to maintain cross-pollination rates below specified levels. The results of some cross-pollination studies may appear inconsistent with the separation distances that have been set. This is because they must be extrapolated for whole field situations (Ingram 2000). Also, in setting separation distances, the variety of the crop used (e.g. whether it contains male sterile plants), the environmental conditions, including the possibility of extreme weather are taken into account. However, even though variability is inevitable, all the evidence suggests that cross-pollination between fields declines very rapidly with distance and that separation distances are very effective in reducing pollen-mediated gene flow to low levels.

The main view of experts in this area is that there are sufficient data available to predict the separation distances required to limit pollen-mediated gene flow to below 1% for most, and below 0.5% for many crop varieties. However, for certain varieties e.g. varietal associations and partially restored hybrids of oilseed rape there is insufficient information to predict separation distances needed to reduce cross-pollination below 0.5% (Ingram 2000).

[5] http://www.soilassociation.org/pollenreport

The typical pattern of decline in cross-pollination over relatively short distances (up to a few hundred metres) may not apply at greater distances (many hundreds of metres to several kilometres), where the pattern is defined by very rare events (Perry, 2002). There have been a small number of studies involving mathematical models that predict gene flow on a landscape scale (Squire *et al*. 1999; Perry 2002). In 2000, a non-GM herbicide tolerant oilseed rape variety was grown for the first time in Australia and this provided an opportunity to study gene flow on a landscape scale without the need for mathematical modelling (Rieger *et al.* 2002). Forty eight million oilseed rape plants were examined within 5km of the source fields. The results showed that, in most cases pollen-mediated gene flow occurred within the source fields - less than 1% of pollination events took place in adjacent fields containing oilseed rape varieties without the herbicide tolerance trait. A small amount of gene flow was detected up to 3km from a pollen source and the distribution of these isolated long-distance pollination events was more variable than would have been predicted from small-scale experiments.

A contributor to the GM Review website has highlighted the results of the Nature paper by and Quist and Chapela (2001). Although the experimental design of this study was flawed (Kaplinsky *et al*., 2002; Metz M. and Fütterer, 2002) it is generally accepted that there has been gene flow between GM maize and maize that is native to Mexico (landraces). However, this is very unlikely to be evidence of unexpected gene flow over extreme distances (i.e. from North America) – it much more probable that cross-pollination has occurred between the landraces and GM plants grown in the same field[6].

Another contributor to the GM review website has highlighted the findings of a report representing the combined findings of two separate Defra monitoring contracts on gene flow from large scale releases of GM oilseed rape between 1994 and 2000 (Monitoring large scale releases of genetically modified crops, EPG 1/5/84. Incorporating report on project EPG 1/5/30: monitoring releases of genetically modified crop plants)[7]. The results showed that with fully fertile varieties, cross-pollination events declined rapidly with distance from the source and most occurred within the first ten metres. However in some cases, cross-pollination levels exceeded 0.5% at distances of 100 – 200 m and the amount of outcrossing associated with varietal associations was considerably higher than that found in samples of fully fertile rape. These results are within the expected range but emphasize the importance of recognising varietal differences when considering separation distances.

What impact do volunteers have on crop to crop gene flow?

Residual seed remaining after a crop has been harvested may germinate in subsequent years producing volunteer plants that can transfer genes to other varieties of the same crop grown at the site. This may be a significant route for gene flow between varieties, for example if oilseed rape volunteers are not controlled it is likely that they could contribute more to impurities in crops than gene flow by pollen movement from other varieties. The length of time that the seed from different crops can remain viable in the soil varies considerably. For example, seed from oilseed rape varieties persists for around six years (although this can be up to ten years in exceptional circumstances) whereas maize seed remains viable for less than a year.

The ACRE's view is that it is good practice, to keep seed shed from GM crops such as oilseed rape and potatoes, on the soil surface and to encourage it to germinate - this allows volunteers

[6] ACRE's advice: http://www.defra.gov.uk/environment/acre/advice/advice14.htm
[7] http://www.defra.gov.uk/environment/gm/research/epg-1-5-84.htm

to be controlled and limits accumulation of seed in the soil. The period before the same crop (either a GM or non-GM variety) can be grown on the same site is assessed. This depends on the potential for seed to remain viable in the seed bank and whether volunteers continue to germinate at the site. Consents for small-scale research and development trials of GM crops require that the sites are monitored after the plants are removed for a minimum number of years, or until volunteers no longer emerge. Removing volunteers before they flower will prevent gene flow to sexually compatible plants.

A view expressed in a contribution to the GM review website is that consents to release GM crops for trial purposes are inadequate from the point of view of volunteer control. The evidence cited was the occurrence of GM oilseed rape volunteers at a site at least four years after the original GM crop was harvested (monitoring large scale releases of genetically modified crops, EPG 1/5/84)[8] and studies that have shown that oilseed rape can germinate 8 years after seed shed and that seeds can remain dormant for around 10 years. It is well known that oilseed rape seed can persist in the soil for these periods and can give rise to volunteers. Therefore in order to prevent these volunteers mediating gene flow, they must be removed before flowering, or at least before seed set if there are no sexually compatible plants in the vicinity.

Gene flow between crop varieties is inevitable, whether mediated through volunteers or not. It can be restricted to low levels but if the GM crops themselves, or gene flow from them, poses a hazard they should not be released into the environment. Gene flow therefore, represents exposure not risk; to assess risk, the potential consequences of crop to crop gene flow must also be considered.

What are the potential consequences of crop to crop gene flow?

Crop to crop gene flow results in transgenes being transferred between extremely similar genetic backgrounds. For this reason the consequences of transgene presence in GM crops will mainly be dealt with in Chapters 4 and 6. This paper considers issues that are unique to plants receiving transgenes unintentionally through cross-pollination.

Pollen-mediated gene flow from GM crop varieties results in transgene presence in the seed of recipient non-GM plants but not in other parts of the plants. Therefore in the first instance, seed crops such as oilseed rape and cereals will contain transgenic DNA as a result of cross-pollination, whereas root crops such as potatoes and sugar/fodder beet[9] will not. However, seed that germinates from these plants will be hybrid and this will contain transgenic DNA in all cells.

'*Transgene stacking'* i.e. the accumulation of transgenes (encoding different traits) resulting from cross-pollination between different GM varieties, was raised during this Review. If several GM varieties of a crop were to be given commercial approval for cultivation in the UK, and were grown widely, then the strong possibility exists that transgene stacking would occur. This might involve transgenes conferring resistance to several herbicides, raising the possibility of multiple herbicide resistance (Orsen, 2001 and Beckie *et al.* 2001), as has happened in Canada - for further details please refer to Dr Linda Hall's presentation to the

[8] http://www.defra.gov.uk/environment/gm/research/epg-1-5-84.htm

[9] Sugar/fodder beet generally flowers in its second year. Roots are harvested in the first year before flowering occurs and therefore seed production is uncommon. Premature flowering can occur and it is good practice to remove these premature bolters before they set seed.

Royal Society discussion meeting.[10] The possibility of generating GM crop plants that are invasive of semi-natural habitats (see section 6.2) as a result of transgene stacking is conceivable in the more distant future, especially if a range of GM crop varieties with resistance to different pests, diseases or other environmental stresses (see section 6.6) are grown on a commercial scale in the UK. However, it should be noted that crops containing such transgenes have not been approved for cultivation in the UK, and are unlikely to be approved in the near future (refer to Chapter 6). In addition, gene stacking is not unique to GM crops. Non-GM varieties have been bred that are resistant to different pests and diseases and have different tolerances to environmental stresses, however the combination of such traits has as yet, not resulted in plants that are invasive of semi-natural habitats.

In Canada, crop varieties with novel traits such as herbicide tolerance fall under the same regulatory framework whether they are GM or not. Once approved, farmers can plant these crops where they choose. In western Canada, farmers have rapidly adopted the use of herbicide resistant oilseed rape and grow the different varieties in close proximity - gene flow between them is therefore inevitable.

The advent of multiple herbicide tolerant volunteers in Canada has necessitated changes in management practices in subsequent crops. There is some concern about the implications that this might have on farmland biodiversity in the UK. In 2002, English Nature published a report that considered what could be learned from the Canadian experience of herbicide tolerant oilseed rape volunteers (Orson, 2002). The conclusion was that if GM varieties of oilseed rape with tolerance to glyphosate (Roundup Ready) and glufosinate (Liberty Link) were introduced into the UK on a commercial scale, stacking of these traits would be inevitable, but that this would have little impact on other agricultural practices. The report suggested that the main implication for herbicide use was likely to be increased usage of paraquat +/- diquat predrilling, which might have an impact on hares. However, studies of European hare populations suggest that this is unlikely to be the case (Edwards *et al.* 2000). The English Nature report recommends that methods for controlling multiple herbicide tolerant volunteers of oilseed rape should be put in place that have minimal or no impact on biodiversity.

ACRE considers the possibility of gene stacking through gene flow and its consequences when assessing the potential impact of releasing a GM crop into the environment. This involves a consideration of what transgenes are present in other GM varieties of the same crop that already have approval for release. Assessing the ecological behaviour of a phenotype that has resulted from the stacking of different traits (in GM or non-GM plants) can be difficult however, as it often relies on evidence other than direct field data. ACRE has the power to require field evidence of the behaviour of novel phenotypes derived from transgene stacking or to invoke the precautionary principle, but such situations have not yet arisen in the UK. There have been some studies that have looked at the potential effects of transgene stacking e.g. Senior *et al.* (2002) deliberately introduced tolerance to glufosinate and glyphosate into the same plants and looked for interaction between them (and did not find any). However, possible transgene combinations must be assessed on a case by case basis irrespective of whether stacking is deliberate or unintentional.

There is a widely held view that transgenes that are used to produce pharmaceuticals or other GM products (e.g. bioplastics and biofuels), that might adversely affect human health if eaten

[10] http://www.gmsciencedebate.org.uk/meetings/pdf/110203-transcript.pdf.

inadvertently, should not be transformed into major food crops since unintended mixing of seed or cross-pollination events could contaminate varieties used as food or feed. The view is that it would be more appropriate to introduce such traits into non-food crops or that the production of pharmaceuticals should be confined to contained facilities and that field releases of such 'pharm crops' should not be allowed. An alternative view is that the most effective way of regulating such crops is on a case by case basis.

To what extent can we detect GM presence?

Our ability to detect and quantify unintended GM presence is fundamental in monitoring gene flow from GM crops and providing consumers with choice. The main analytical methods either target DNA (e.g. the polymerase chain reaction [PCR]) or the products of DNA expression (e.g. enzyme linked immunosorbant assays [ELISA] and immunochromatographic strip tests for proteins). The distinction between these two approaches is significant as transgenic DNA inserted into crop genomes is not necessarily expressed and translated into protein. Gene expression may be very low or non-existent in different organs of a plant or at different stages in its development. Therefore, tests for transgenic protein in a sample may be negative whereas DNA analysis demonstrates that transgenic DNA is present.

Currently, methods involving PCR predominate in detecting and quantifying DNA – these are very sensitive and can be used to detect DNA that is present at very low abundance (the threshold for detection is different for different crops). However, it is generally accepted that a minimum of 0.1% GM presence is required (i.e. 1 transgenic seed in 1 000 non transgenic seeds) for detection to be reliable. Detecting the presence of transgenic DNA in processed foods is potentially more difficult. This because the DNA may be damaged, or other constituents in the foodstuff may interfere with its detection. In the case of some highly refined products such as sugar and oil, a lack of DNA or protein means that it is impossible to determine whether the crop was GM or not. In these cases, authenticated audit trails would be necessary.

In order to enforce a threshold level of unintended GM presence, PCR techniques must also be able to accurately quantify transgenic DNA. The accuracy of measurements decreases at lower thresholds of transgenic DNA presence due to error associated with sampling and also with the PCR itself. Therefore increasing sample size (e.g. the number of seeds or tubers) reduces sampling error and increases the confidence in the accuracy of transgenic DNA measurements. However, to achieve accurate quantification at lower thresholds of GM presence the sample size must increase dramatically (Kay and Van den Eede, 2001). Sample sizes are likely to limit detection thresholds much below 0.1%.

The amount of pollen-mediated gene flow between different varieties, particularly those separated by bare ground or low vegetation, is likely to be significantly higher at the edge, than in the middle of the fields. Therefore, plants along the field edge may have higher than threshold levels of GM presence whereas the remainder of the field may have significantly lower levels.

Directive 2001/18/EC (for the release of GMOs in the EU) requires that information for the identification of individual GM varieties is made available for monitoring purposes i.e. DNA sequences specific to each transgenic crop variety (e.g. junction fragments that span the intersection of inserted transgenic DNA with native host DNA). Therefore, unintended presence of approved GM crop varieties that have gone through statutory regulatory

assessment in the EU may be identified. However, there are other classes of GM crops where this detailed molecular information is not readily available e.g.

- GM plants released for small-scale research and development trials (under part B regulatory approval).

- GM material that has not been through the EU regulatory process. Seed for use in the UK is frequently multiplied abroad because it makes it possible to obtain more than one generation of seeds per year. For example, some maize varieties grown on organic farms in the UK are multiplied in North America where gene flow from GM varieties that have not been approved in the EU could take place. In contributions to the review, concern was expressed about an instance where non-GM oilseed rape seed imported from Canada by Advanta Seeds UK Ltd was found to contain about 1% of a GM oilseed rape variety that was approved for food, feed and environmental release in Canada but not in Europe. There are international databases containing information on transformation events that have been approved around the world (e.g. the OECD's Biotrack online[11]), and links between them are being developed to give a more integrated resource. However, these do not contain the detailed molecular data that must be registered by applicants seeking approval to release GMOs commercially in Europe (see above). GMOs that have not received consent for release, or are not in the regulatory process, will generally be the most difficult to detect, and more particularly, to identify.

Screening for genetic elements commonly used in GM crops (e.g. the cauliflower mosaic virus [CaMV] 35S promoter) may identify unintended GM presence in some cases - however, this is not reliable since these elements are commonly found in nature (results in false positives because the DNA is derived from a source other than the crop), or the transgenic DNA present does not contain them (results in false negatives because different transgenic elements have been used).

To what extent can gene flow be contained by genetic isolation systems in crop plants?

There is an interest in developing mechanisms that could prevent or restrict gene flow from GM crops [so-called 'genetic use restriction technologies' (GURTs)]. There are a number of potential ways in which at least partial genetic isolation might be achieved. Some of these are established technologies whilst others require considerable research and development. There are also systems that have not necessarily been developed for this purpose but which also affect the transfer of genes from GM crops. Plastid transformation and so called 'terminator technologies' have been highlighted for the attention of this review:

- Insertion of transgenic DNA into chloroplasts rather than the nuclear genome has been proposed as a method for minimising gene flow (Daniell *et al* 1998). This proposition has stimulated considerable debate (e.g. Daniell and Varma, 1998; Chamberlain and Stewart, 1999). Firstly, it relies on the assumption that chloroplasts are always maternally inherited in crops. The mode of chloroplast inheritance is known for the majority of cultivated species but can be influenced by both genetic and environmental factors (Stewart and Prakash, 1998). In addition, a recent paper by Huang *et al.* (2003)

[11] http://www1.oecd.org/scripts/biotech/frameset.asp

indicated that gene flow from chloroplasts to the nucleus can occur at higher frequencies than previously supposed. However, this study used highly selective laboratory conditions that would not be present in the field. Nevertheless, plastid transformation could still significantly reduce the potential for gene flow by pollen-mediated hybridisation events, although it could also increase the probability of horizontal gene transfer (HGT) occurring due to increased copy number and similarity between plastid genomes and those of soil and gut microbes

- Several companies have developed strategies that make use of promoters, inducible by chemical stimulants, to regulate the expression of transgenic proteins that interfere with anther development or seed germination. This allows for seed multiplication but means that hybrid seed generated from the crop would be unviable or the resultant plants would be male sterile.

In addition to these, there are a number of other mechanisms that could prevent or reduce pollen-mediated gene flow from GM crops. These are based on systems that occur in nature and include: *Apomixis*: the production of seeds without fertilisation; *Cleistogamy*: flowers are produced that develop normally, but fail to open and the *exploitation of hybridisation barriers*. Alternatively the desired transgene can be coupled with genes that would render hybrid offspring or volunteers less able to compete with crops, weeds and wild species. Genes that prevent seed shatter or secondary dormancy, or that dwarf the recipient could all be useful for mitigation. Many such genes have been isolated in the past few years (Gressel, 1999). ACRE has issued guidance on the development of mechanisms in GM crops that could minimise transgene dispersal in its *Guidance on Principles of Best Practice in the Design of Genetically Modified Plants*[12].

The exploitation of differences in flowering time between varieties may restrict gene flow between them, but it is unlikely to be sufficiently reliable to prevent it. The use of crops with no sexually compatible semi-natural or weedy relatives in the UK, provides a simple and effective way of containing transgenes within crop plants.

In a contribution to the GM science review, the use of site-specific recombinases in constructs designed to prevent gene flow from GM crops was raised. The concern expressed is that these recombinases will cause DNA rearrangements (Mae-Wan Ho and Joe Cummins)[13]. Recombinase methods are not yet well developed, but refinements in technologies to excise transgenes or parts of inserted DNA are likely to become available in the future (Hare and Chua, 2002).

Is co-existence between different agricultural systems possible?

'*Farmers and consumers alike are concerned about the freedom of choice of different agricultural production systems. In my understanding, co-existence means that no form of agriculture, GMO or non-GMO, should be excluded in the EU in the future. Similarly, it is also linked to consumer choice. Only if farmers are able to produce the different types of crops in a sustainable way, will consumers have a real choice'* (Franz Fischler, member of the EU commission, 2003)[14].

[12] http://www.defra.gov.uk/environment/acre/bestprac/guidance/index.htm
[13] http://www.gmsciencedebate.org.uk/topics/forum/0046.htm
[14] http://europa.eu.int/comm/research/biosociety/pdf/rt_fischler.pdf

Strategies for co-existence of GM, conventional non-GM and organic crops are already in place in countries worldwide, even in Europe. For example, in Spain there has been successful cultivation of Bt maize over the past 5 years with the utilisation of cost-effective good agricultural practices. However, the experience of other countries may not be directly relevant and co-existence must be considered on a crop by crop basis in the UK. The European Union held a round table meeting with various stakeholders in April 2003 and are expected to deliver guidance on co-existence in July 2003. Gene Flow and co-existence in oilseed rape and maize was discussed at the open meeting held in Aberystwyth[15]. These crops were selected for discussion as both are open-pollinating crops with the ability to disperse genes quite widely from crop to crop and also because they are about to be commercialised, or are already commercialised as GM crops in Europe.

In May 2002, the European Commission published a study by their Joint Research Centre (JRC) entitled: Scenarios for co-existence of genetically modified, conventional and organic crops in European agriculture (Bock *et al.* 2002)[16]. This used computer modelling and expert scientific opinion to analyse the practicalities and costs of achieving co-existence for three crops (potatoes, grain maize and oilseed rape) in various hypothetical scenarios (thresholds for incidental GM presence of 0.1%, 0.3% or 1.0% with GM crops as 10% or 50% of the total cropping). The report concludes that co-existence around a 0.1% threshold would be very difficult, if not impossible for the crops considered. It suggests that in some cases, existing farming practices will be sufficient to achieve a 1% threshold. The report is generally considered to be a useful first step towards assessing the consequences of the introduction of GM crops on a commercial scale in Europe and in identifying appropriate measures at the farm level to minimise the unintended presence of GMOs below the legal thresholds laid down by the Commission but that it shouldn't be taken as an anticipation of future developments.

More recently a study by an expert working group in Denmark (Tolstrup *et al.* 2003) on '*the co-existence of GM crops with conventional and organic crops*' was published. The study concluded that if there was limited GM-production (10%) and a threshold of 1 % for unintended GM presence in non-GM crops, co-existence could be maintained for most crops in Denmark (i.e. beet, maize, potatoes, barley, wheat, oats, triticale, rye, lupine, broad beans and peas), although, for some of these crops current farming practices might need to be modified. For oilseed rape, as well as for seed production of certain crops, the working group suggested that reliably maintaining co-existence could be more problematic and suggested that further evaluation would be required, before guidelines could be developed.

The feasibility of establishing a separate supply and production chain for GM and non-GM crops is dependent on our understanding of the crops themselves and how the genes move. There is a substantive body of scientific evidence indicating that restricting GM presence (or non-GM presence in the case of GM crops) to very low levels is relatively straightforward for some crops, whereas for others, some alteration in farming practice is required. For example, it will be particularly difficult to achieve a very low threshold of GM presence in farm-saved oilseed rape seed on farms where both non-GM and GM oilseed rape varieties are grown. Ultimately however, the threshold levels that are set will determine whether co-existence is practical in the EU. The AEBC will shortly be publishing a report entitled: '*GM crops, coexistence, choice and redress*', and this looks at how far it would be practicable for the

[15] http://www.gmsciencedebate.org.uk/meetings/pdf/170303-transcript.pdf
[16] http://www.jrc.es/projects/co_existence/Docs/coexreportipts.pdf

commercial production of GM crops to co-exist with conventional and organic systems of agricultural production.

7.2.4 Is there general scientific agreement?

There is general scientific agreement that gene flow from GM crops will occur, although this will differ significantly depending on the crop and on the variety in question. The release of GM crops is regulated and the potential consequence of gene flow is a component of a detailed risk assessment. The vast majority of gene transfer occurs within a relatively short distance of its source. The use of separation distances and agricultural practices that limit gene flow will enable the unintended presence of transgenic DNA to be maintained at low levels for most crop varieties. There are many additional political and economic issues that will determine whether co-existence is ultimately possible – this includes what the maximum legal thresholds of GM presence in non-GM crops and their products will be.

The consensus view is that the minimum GM presence that can be achieved is dependent on the variety not just on the crop. For most major crops gene flow can be restricted to at least 1% and for a number it can be far less. However, as the probability of rare cross-pollination events can stay more or less constant for several kilometres for some crops, separation distances will not ensure genetic isolation.

There is some disagreement about whether the separation distances that are currently used to restrict gene flow from GM crops in research and development trials are sufficient. These thresholds have been extrapolated from a substantial range of available data that has been applied to whole field situations.

The potential error associated with quantifying transgenic DNA when it is present at very low levels limits the threshold levels of GM presence that can be accurately quantified and therefore regulated – as opposed to the sensitivity of the technology itself. The international adoption of validated sampling as well as analytical methods will be important in monitoring for unintended GM presence.

The registration of DNA sequences that are unique to particular GM crop varieties with approval for commercial release in the EU will facilitate their detection and identification. However, GM presence arising from commercial varieties that do not have EU approval will be more difficult to identify.

7.2.5 Are the issues unique to GM?

Gene flow between different varieties of the same crop is almost as old as agriculture itself. The desire to restrict gene flow is not unique to GM agriculture either. If GM crops are commercialised, restricting gene flow between non-GM and GM varieties will potentially be on a much larger scale than anything that has preceded it. Gene flow would be restricted because of legal thresholds and consumer demand rather than necessarily risk management. Gene stacking is not unique to GM, but it is possible that it could result in combinations of traits that would not occur in non-GM crops.

7.2.6 Are there gaps in our knowledge or scientific uncertainties and are these important?

We know a great deal less about long distance gene flow than we do about gene flow over distances of a few hundred metres or less. The consequences of this are more significant for some crops than others (e.g. those that have a greater tendency for cross-pollination such as oilseed rape varieties that contain male sterile plants). Such data has been difficult to obtain before the advent of GM crops except in unique situations such as that in Australia when non-GM herbicide resistant oilseed rape was introduced for the first time recently (Reiger *et al*. 2002). However, it is debatable whether more research on long distance gene flow will provide us with more useful information apart from reinforcing the conclusion that it occurs at very low frequencies and is variable.

In the main, gene flow between crop varieties has not been studied on a farm or regional scale (however, there are exceptions: e.g. Squire *et al.* 1999) - models have been developed but these are largely based on studies carried out on a much smaller scale. If GM crops are grown commercially in the UK, monitoring gene flow as the scale of their introduction increases will be important in refining our predictions.

A large area of uncertainty is the way in which the different factors in determining co-existence will combine at a commercial scale – i.e. the real-life consequences of the combination of adventitious presence in seed, cross-pollination, and the contribution of volunteers.

It important that farmers and others involved in supplying GM crops are provided with accurate guidance on management practices that restrict seed-mediated gene flow.

Advanced diagnostic and sampling methodologies for determining the extent of gene flow early in the production/ supply chain will be important in facilitating co-existence.

7.2.7 Likely future developments

It is unlikely that further studies on gene flow from crop varieties will allow us to reduce thresholds of maximum GM presence any further. However, in the longer term it is possible that gene containment systems will be developed that will significantly, if not totally reduce gene flow. If the complete genetic isolation of a GM crop variety is to be achieved, it is likely that it will need to contain a combination of systems that prevent seed, as well as pollen-mediated gene flow. However, these mechanisms are likely to introduce new complications for those developing such varieties and producing seed on a commercial scale.

Several strategies have, and are being developed to eliminate selectable marker genes from plant genomes after transformation, or to control fertility (e.g. site specific recombination, homologous recombination, transposition and co-transformation – Hare and Chua, 2002). Marker removal may be desirable for functional, economic, regulatory, or perhaps safety reasons. Fertility control might be used to produce hybrid seed, prevent pollen-mediated gene flow, stop pollen production for allergy or energy reasons, or prevent seed production for regulatory or commercial reasons. Recombinase methods are not yet well developed, but refinements in technologies to excise transgenes or parts of inserted DNA are likely to become available in the future.

Plant scientists working on the development of RNA interference technology (affects levels of gene expression) consider that this, along with our increasing understanding of the function of different plant genes will provide an opportunity to develop a range of GM crop varieties containing gene constructs based on native plant genes as opposed to transgenes from other organisms.

However, future generations of GM crops may also be used as 'biorefineries' for making novel products such as biofuels and pharmaceuticals.The existing practice of assessing each new GMO on a case by case basis is appropriate for regulating these new types of GM crops.

Transgene stacking will become more likely if a number of different GM crop varieties are grown on a commercial scale. This will provide challenges to the regulatory system in assessing the implications as it will involve the unintentional combination of genes in the countryside that will not have been combined deliberately during the development of the GM variety. The more GM varieties of the same crop that are grown commercially, the larger the potential combinations of transgenes combinations to be considered. The stacking of GM traits is not the only issue that regulators consider – the possibility that transgene products might interact at a biochemical level is also assessed.

Advanced diagnostic and sampling methodologies for detecting GM presence in non-GM crops are being developed. Approved methodologies for detecting, quantifying and identifying GM presence at different stages in the supply chain will be important in maintaining co-existence.

7.2.8 Where there is important scientific uncertainty, what is the potential way forward?

Research

Research into gene flow on a larger scale is being undertaken. This involves a project to measure outcrossing from fields in Scotland that are sown with the GM herbicide-tolerant crops of oilseed rape used in the farm-scale evaluations. The easily detectable markers in these crops should allow more accurate estimates of cross-pollination at low frequency than had been possible before. For wider applications, a consortium led by SCRI is developing advanced, high throughput diagnostic techniques for measuring gene flow at low frequency among non-GM fields. Together these studies will also quantify the pollination efficiency of insects, such as bumble bees, hive bees and pollen beetles that contribute to crossing, quantify the spatial patterns of crossing in fields, and develop the sampling protocols necessary to estimate whole-field crossing accurately.

If GM crops are to be grown on a commercial scale in the UK, monitoring gene flow as the introductory process develops will be important in ensuring that measures to maintain co-existence are working and that further steps do not need to be taken. Assessing the relationship between crop-to-crop gene flow and the legal thresholds for GM presence in non-GM food chains is a key aspect of gene flow research.

Technological/ agronomic approaches

It is important that there are accurate guidelines on management practices that restrict seed-mediated gene flow and that farmers and others involved in producing and supplying seed implement them.

Current sampling and detecting methodologies must be capable of supporting legislation on maximum GM presence thresholds. To this end there must be internationally approved monitoring, sampling and detection methods for all crops (and products derived from them) that are capable of facilitating the detection and quantification of GM presence at, or below any threshold levels that are set. These are currently being developed in a collaborative effort involving a number of European laboratories.

Regulatory approach

The registration of DNA sequences that are unique to particular GM transformation events used to develop GM crop varieties, approved for commercial release in the EU, will facilitate their detection and identification. However, GM presence from varieties that do not have EU approval will be more difficult, if not impossible to detect. Information about transformation events that have been approved outside of the EU is widely available but this does not include the detailed molecular data available for EU approved events.

Currently, continuing assessments of the consequences of gene flow from GM crops are made on a case by case basis. This is the most effective way of dealing with GM crops with novel traits. Regulators will have to continue to be mindful of the possible consequences of transgene stacking.

7.3 GENE FLOW FROM GM CROPS TO AGRICULTURAL WEEDS AND WILD RELATIVES

Can the extent and consequences of gene flow from GM crops to agricultural weeds and wild relatives be predicted and controlled? Could gene flow from GM crops generate superweeds or eliminate wild plant populations?

7.3.1 Summary

Gene flow – the transfer of genes, in pollen or seed, from one population to another - is commonplace among closely related adjacent plant populations. Gene flow by cross-pollination involves both hybridisation and the incorporation of the gene into the new population (introgression). This last process varies greatly from one situation to another and provides most of the uncertainty in predicting actual amounts of gene flow.

Most modern crops have been bred from wild plants. Nearly all hybridise with one or more wild relatives somewhere in the world, but modern agriculture has moved many crops outside the range of sexually compatible wild relatives. Crop-to-wild relative gene flow varies between different crops and different regions. For example in the UK gene flow to the wild is not an issue for crops such as wheat, maize, potatoes and tomatoes but must be considered for those such as ryegrass, clover, sugar beet and oilseed rape.

The exchange of genes between crops and their wild relatives that has occurred during the long period of crop domestication continues today, often aided by farmers in small-scale agriculture. This and the movement of seed around the world has made it difficult to measure accurately in specific cases the rates of contemporary gene flow. Recent studies using molecular methods are providing new insights into these rates.

If hybridisation and introgression occur, the subsequent spread of the gene could be increased by continuing high rates of gene flow, the gene's accidental fixation in small populations or the overall greater fitness of wild plants with the gene than those without it. An increase in the gene in the wild relative does not necessarily mean it will become more persistent or invasive – other ecological criteria discussed in section 6.2 apply to invasiveness.

Modern studies (particularly on beet and oilseed rape) have confirmed that gene flow to wild relatives occurring as weeds in arable fields and disturbed agricultural habitats is higher than to wild relatives occurring in semi-natural environments. Gene flow rates also very considerably from place to place depending on a range of conditions. As expected gene flow mediated by seed transfer to semi-natural situations has been demonstrated (in sugar beet) but gene flow by cross pollination and subsequent introgression appears generally lower in these environments. This is believed to be due to selection during domestication of traits which are disadvantageous in the wild.

More than two decades of experience with the technology indicates that in the context of gene flow transgenes behave exactly as resident naturally-occurring genes. The issue of gene flow from crops to wild relatives is not unique to GM, and there is no evidence that current transgenes are more likely to transfer or persist in the wild than other genes. However, each crop/gene combination is, and must continue to be, considered on a case-by-case basis.

There is broad consensus that, in particular cases, gene flow to wild relatives is inevitable and that gene flow itself is not intrinsically harmful. It is the consequences of gene flow that are important. For example genes conferring herbicide tolerance have the potential to create an agricultural weed management problem, especially if weed tolerance of more than one herbicide occurs by gene stacking. On the other hand, herbicide tolerance has been shown to be at best neutral, and sometimes disadvantageous in wild plants and situations where herbicides are not applied. Genes conferring resistance to insect pests or pathogens have the potential to increase the fitness of a wild relative. Again, however, this possibility must be examined on a case-by-case basis.

Most of the gaps in our knowledge of gene flow relate to its consequences. Whilst genes for pest and disease resistance introduced into crops by conventional breeding have not produced invasions of wild relatives in semi-natural environments, current regulatory oversight of GM crops deals with this possibility on a case-by-case basis. In those cases where gene flow is possible, however rare or improbable, the consequences are assessed. Consent to release a GM crop would not be given were any harm to human health or the environment envisaged from the transfer of a transgene by gene flow to wild relatives.

7.3.2 Background

Gene transfer from GM crops to agricultural weeds and wild relatives has been addressed in two open meetings associated with the GM Science Review: the Royal Society of Edinburgh Meeting in January 2003 and the scientific discussion meeting at the Royal Society in February 2003. Abstracts and transcripts of these meetings are available on the GM Science Review website[17]. The GM Science Review has also received a number of contributions to its website about gene flow from crop plants to agricultural weeds and wild relatives. These have presented evidence (e.g. Chris Lamb)[18], concerns (e.g. Michael Cates)[19] and questions (e.g. GeneWatch)[20].

Gene flow is the transfer of genes, in pollen or seed, from one population to another and the incorporation of those genes into the gene pool of the recipient population (Futuyama, 1998). In the case of pollen transfer, it is essentially a two-stage process: hybridisation and introgression. For hybridisation to occur the plants must be sexually compatible and flower at the same time, viable pollen must be delivered to the stigma and successful fertilisation of the embryo must be followed by zygote and seed formation. Introgression requires the hybrid seed to germinate and the (F_1) plant to establish and flower in order to further hybridise with members of the recipient population.

Such gene flow is commonplace among closely adjacent populations of the same species, although in many species it can be reduced by self-fertilisation or various inherited incompatibility systems. The amount of gene flow reduces with increasing physical distance of populations of the same species and with increasing evolutionary distance of different species, i.e. decreasing relatedness. Although to some extent all plants are related, the

[17] http://www.gmsciencedebate.org.uk/meetings/default.htm
[18] http://www.gmsciencedebate.org.uk/topics/forum/0064.htm
[19] http://www.gmsciencedebate.org.uk/topics/forum/0015.htm
[20] http://www.gmsciencedebate.org.uk/topics/forum/0007.htm

presence and strength of the various barriers to hybridisation outlined above is determined by how closely related the species are. Whilst modern plant breeding uses a range of mechanisms to overcome these barriers (e.g. embryo rescue, GM), in the field the range of related species with which a crop may hybridise is specific for each crop species and is known for (almost) all of them. The extent to which introgression can occur however, varies greatly between species (and situations), and, by contrast, is less well known. This element of gene flow, introgression, is therefore responsible for most of the uncertainty in determining or predicting actual rates of gene flow. It is also influenced by the effect of the transferred gene(s) on the plant (specifically plant fitness). Gene flow and its consequences are thus intimately confounded.

Most crops have been bred from wild plants. On a global scale it is therefore not surprising that nearly all crops may hybridise with a wild relative in some part of their distribution range (Small 1984, Ellstrand *et al* 1999). However only a tiny fraction of the world's flora has been domesticated and in modern agriculture many crops are grown outside the range of the wild relatives, often their antecedents, with which they might hybridise. The potential for gene flow from crops to wild relatives will therefore vary from region to region. In the UK, for example, gene flow is not an issue for crops such as wheat, maize, potatoes and tomatoes (because they have no sexually compatible wild relatives in the UK) but is a major issue for, among others, ryegrass, clover, sugar beet and oilseed rape (Raybould & Gray 1993). (In Europe an analysis of possible gene flow has been made for the flora of the Netherlands (de Vries *et al* 1992), Switzerland (Jacot & Ammann 1999) and the UK (Raybould & Gray 1993)). The two crops for which applications for commercial release have been made where gene flow to wild relatives must be considered are oilseed rape (*Brassica napus*) and sugar/fodder beet (*Beta vulgaris*). These are discussed in more detail later.

In the context of gene flow three types of 'wild' plant populations can be distinguished. These are:

(i) **Feral Populations** – crop plants which have, perhaps temporarily, escaped from cultivation and are growing in the wild, often in habitats which are frequently disturbed. Oilseed rape on road verges is perhaps the most familiar current example but several crop species have established feral populations in the British countryside [e.g. lucerne, chicory, carrot (Stace 1991)].

(ii) **Weedy relatives** – species related to crops which may grow within the crop, sometimes becoming weeds ('plants in the wrong place'), or in peri-agricultural environments (tracks, verges, headlands etc). Examples include wild turnip, charlock and weed beet.

(iii) **Relatives growing in 'natural' environments** – plants which occur outside arable agriculture in the UK's semi-natural habitat-types such as chalk grassland, heathland, saltmarsh or woodland. These include clover, ryegrass, wild cabbage and sea beet.

Some species may occur in more than one category. For example both wild turnip (*Brassica rapa*) and wild radish (*Raphanus raphinistrum*) have populations that occur in agricultural environments and other populations (possibly subspecies) which are found in semi-natural habitats (*B.rapa* on riversides, *R.raphinistrum* in sand dunes). Other species, such as wild cabbage (*Brassica oleracea*) on coastal cliffs, may have

originated as feral populations many years ago, but are not weedy and are now regarded as naturalised (Preston *et al* 2002).

For any particular crop, on a case-by-case basis, it is necessary to assess the likelihood and consequences of gene flow to all wild relatives in all of the above three categories (and also to plants 'escaping' from crops e.g. 'volunteers' in oilseed rape, 'groundkeepers' in potatoes, 'bolters' in sugar beet). However in practice concerns about gene flow have usually made a distinction between gene flow to agricultural weeds and gene flow to wild relatives in semi-natural environments. In the first case concern has been largely centred on the possibility of creating agricultural problems such as more herbicide-tolerant weeds (so-called 'superweeds') (Hall et al 2000, Orson 2002). In the second case concerns have included the possibility of the wild plants becoming more persistent or invasive following transfer of a gene which increases their 'fitness', the potential impact on other plant and animal species, and the genetic 'pollution' of natural populations with genes derived originally from sources such as bacteria or viruses (Genewatch 1998, Hill 1999, Daniels & Sheail 1999).

During their long period of domestication many crops have hybridised with wild relatives, and *vice versa* (DeWet & Harlan 1975, Pickersgill 1981). Farmers have often selected these hybrids for cultivation, and in some crops, especially those under small-scale agriculture (which equals 40% of world agriculture) continue to do so (Jarvis & Hodgkin 1999). These crops include maize, rice, chillies, potato, sorghum, squash and pearl millet; in the last of these cultivated and wild forms are known to have exchanged genes for at least 3,000 years (Renno *et al* 1997). Many plant species can be found both as a crop and a weed (e.g. oilseed rape) and close relatives may 'mimic' crops under certain forms of agriculture (e.g. rice (Barrett 1983). Furthermore, past agriculture and the wide exchange and movement of seeds has transferred plants, and their genes, to many parts of the globe. For example thousands of tons of white clover seed were imported into Britain from many European countries in the eighteenth and nineteenth centuries, from North America as long ago as the beginning of the nineteenth century and from New Zealand in more recent times (making it difficult to define a 'native' genotype) (Gray *et al* 2003).

All these factors, the common evolutionary lineage, the link in various combinations between crops, weeds and wild relatives, the (frequently unknown) movement of seeds, mean that modern crops and their wild relatives often have many genes in common (technically they are said to share genes by descent). This has made it very difficult, at least until recently and the advent of molecular methods, to quantify the amounts of contemporary gene flow. In other words we know that gene flow has, or could have, happened but we cannot usually say with any accuracy how frequent it is today. However some studies, described below, are beginning to provide estimates of gene flow rates for specific crops.

Among the factors that are known to affect the amount of gene flow (relatedness/hybridisation barriers, degree of self-pollination, and so on), there is a great deal of information on the effect of distance. Cross-pollination falls off rapidly with distance but the distance at which it is zero is impossible to determine with accuracy. Curves describing the frequency of cross-pollination at various distances from a pollen source have been derived from experiments and used particularly to calculate the separation distances required between GM and non-GM crops in order to achieve minimal levels of crop-to-crop gene flow (Ingram 2000, Champolivier *et al* 1999). The relative size of the donor and recipient populations is also known to be an important factor (Squire *et al* 1999). In general the large amounts of crop pollen compared to that produced by the (normally) smaller feral or wild populations will tend

to increase gene flow to these.

Providing hybridisation and introgression are possible, genes from crops may theoretically increase in frequency in local wild populations under three conditions. These are –

(iv) Very high levels of cross-pollination giving a constant immigration of crop genes to the population (swamping),

(v) The 'accidental' fixation of the crop gene in a small wild population (genetic drift) and/or,

(vi) Where the gene confers greater lifetime fitness on the individuals with the gene than those without it (selection).

Genes may spread in a population under these conditions, including where the plants containing them are fitter, but this does not mean that the plant populations will become more persistent or invasive – the criteria for this to happen are discussed in section 6.2. It could mean that the wild species becomes genetically more uniform, or depauperate (genetic erosion). This is unlikely unless wild populations are exposed to gene flow from the crop across most of the geographical and ecological range of the species.

In assessing the risks from gene flow to wild relatives the ACRE consider both exposure (the probability of gene flow) and hazard (the harm that might result from gene flow). If it is known that a wild or weedy species is sufficiently sexually compatible for gene flow to occur, however rarely, it is assumed that it will happen (i.e. probability = 1) and the consequences are assessed. Were any harm envisaged, ACRE would advise against issuing consent (Gray 2002a). In cases where partially compatible wild relatives only occasionally co-occur with the crop, information on hybridisation rates may contribute to the risk evaluation; but, again, a transgene which was thought to have potentially harmful effects in that wild relative would not be released.

7.3.3 Range of views and quality of evidence

There is a range of views on the importance and consequences of gene flow from GM crops to agricultural weeds and wild relatives. These include – views about gene flow itself, views about the likelihood and rate of gene flow, and views about the impact and consequences of gene flow, both on agricultural weeds and wild relatives in semi-natural environments.

In addition to the fundamental view (mentioned earlier) that DNA originally derived from bacteria or viruses should not be transferred to wild plant populations, there is a view that their method of insertion in the plant (whether by bacterial plasmid vector or biolistics) makes the behaviour of transgenes unpredictable when inserted in the genomes of wild relatives (the issue of transgene stability is covered in Chapter 4). There is a further view that transgenes differentially interact with native genes in wild relatives under different environmental conditions and that this is not sufficiently understood (N. Rajanaidu – contribution to Review website)[21]. There is a general concern that genes from domesticated plants (including crop

[21] http://www.gmsciencedebate.org.uk/topics/forum/0003.htm

plants and those bred for amenity use) threaten the genetic integrity of local adapted populations and the patterns of genetic diversity within wild species (see Gray 2002b for a review). Whether gene flow from GM crops has additional implications for the genetic identity of these populations is an issue that has been raised during this review (GeneWatch)[22]. Another contributor to the review website expressed a more specific concern about the possible consequences of gene flow from GM crops containing transgenes that confer male sterility (Mae-Wan Ho and Joe Cummins)[23]. Another viewpoint is that the transgenes so far inserted in crops can be viewed and evaluated in essentially the same way as any novel genes and their transfer to weeds and wild relatives is not fundamentally different from the process of gene exchange between crops and relatives which has been occurring for thousands of years (see above). This last view, which is probably the majority of scientific opinion, argues that gene flow to wild relatives is not intrinsically harmful but that every transformation (each crop/gene combination) should be examined on a case-by-case basis to assess whether gene flow may have harmful consequences to human health or the environment.

Evidence that transgenes are inherited and transferred between individuals in a similar way to resident genes may be derived from more than a decade's experience with the technology. Genes inserted by recombinant DNA technology and selected for plant breeding programmes demonstrate Mendelian segregation and recombination and 'flow' from plant to plant exactly as resident genes. Experiments in which crosses have been made between transgenic crops and wild relatives show segregation patterns consistent with the expectation that transgenes are inherited in the same way as naturally occurring genes (Snow *et* al 1999, 2003, Halfhill *et al* 2002 - in these examples for herbicide tolerance and Bt in *Brassica* and Bt in sunflowers). Evidence that past gene flow has had an impact on the population biology and survival of wild species in the UK is difficult to find, although the potential impact globally of modern crops on local land races of several species is a widely acknowledged problem and the presence of genes derived from crops has been established in a number of wild species (e.g. sunflower (Linder *et al* 1998). An example of hybridisation threatening the genetic integrity of a native species is given by Al Mazyad & Amman (1999) who describe gene flow from Lucerne (*Medicago sativa*) to tetraploid populations of sickle medic (*M.falcata*) in regions of Switzerland. Indirect evidence of past gene flow may also be inferred from current patterns of population differentiation, as in rye grass (Warren *et al* 1998).

There is general agreement that in specific cases gene flow to sexually compatible wild relatives will occur. Disagreements are principally quantitative in nature – how much? And how far in specific situations? The evidence from studies of cross-pollination experiments and from crop-to-crop gene flow using marker genes indicates variation in specific cases but the generality of the pollination curve has been established. This shows a rapid decline in cross-pollination after the first 10 to 20 (-50) metres from a pollen source with a low level of cross pollination continuing often over considerable distances (in excess of 500m) (Champolivier *et al* 1999, Rieger *et al* 2002). Such curves vary in detail from species to species depending on their breeding system and their mode of pollination (wind versus insect) but their general form (technically described as leptokurtic) confirms that (a) most plants mate with near neighbours or themselves and (b) rare cross-pollination events occur at long distances. These data suggest that for most crops with wild relatives, as well as those whose relatives co-occur as weeds of agriculture, it will not be possible to prevent cross-pollination. (There are exceptions, e.g. gene flow from lettuce in the UK could be prevented by growing the GM

[22] http://www.gmsciencedebate.org.uk/topics/forum/0007.htm
[23] http://www.gmsciencedebate.org.uk/topics/forum/0046.htm

crop outside the rather restricted range of its sexually compatible wild relative – Raybould & Gray 1993). Empirical evidence that levels of hybridisation are extremely low except where the crop and wild relative occur together (i.e. are separated by less than 2-5m) has been provided for oilseed rape hybridising with wild turnip (*B.rapa*) on river banks (where it is known as Bargeman's cabbage). Using a combination of remote sensing and genetic analysis, Wilkinson *et al* (2000) detected only a single hybrid in *B.rapa* populations in a 15,000 km^2 area of S.E. England. This low number was largely because few wild turnip populations occur next to (within one or two metres) oilseed rape fields. The work is continuing (funded by a BBSRC/NERC gene flow initiative) and has been extended to the rest of the UK, to provide an estimate of annual hybrid production. For *B.rapa* as a crop weed, work in Denmark (Jorgensen & Andersen 1994) has confirmed earlier studies (in 1962 – Palmer) that the highest number of hybrids (80%+) are produced when small numbers of the (self-incompatible, diploid) *B.rapa* are placed in oilseed rape fields (i.e. there is a large excess of oilseed rape pollen).

The likelihood of stable introgression of transgenes into wild populations depends critically on the survival of subsequent generations. Here there is evidence of differences between gene flow to relatives which are arable weeds and gene flow to relatives growing in semi-natural environments. For example, in Denmark, where both oilseed rape (*B.napus*) and wild turnip (*B.rapa*) occur together as weeds in set aside land or organic farmers' fields, substantial introgression beyond the F_1 stage with back-crossing involving both species has occurred. This is supported by clear molecular evidence (Jorgensen *et al* 2003). This contrasts with the potential rates of gene flow to *B.rapa* in semi-natural habitats (i.e. beyond the very low numbers of F_1 hybrids) described above. Similarly, studies in northern France on gene flow between sugar beet (*Beta vulgaris ssp vulgaris*), weed beet (*B.vulgaris ssp. Vulgaris*) and sea beet (*B.vulgaris ssp maritima*) have demonstrated high levels of gene flow between the crop and the (annual) weed beet populations in heavily infested sugar beet fields (Desplanque *et al* 2002) but detected little or no gene flow between sugar beet and nearby sea beet populations (Cuguen 2003). In particular a gene removing a requirement for vernalisation (leading to an annual life cycle) does not appear to have been transferred to sea beet populations in N.France and the UK despite a long exposure of the wild plant to crops and weeds containing the gene Cuguen 2003, Van Dijk *et al* 1997). A recent paper from the Lille group has confirmed that gene flow to habitats where sea beet occurs can be seed mediated (in this case by the transfer of soil) (Arnaud *et al* 2003).

The contrast between rates of gene flow to arable crop weeds and to wild relatives supports the general view that genes transferred from domestic to wild species produce hybrids with poor survivorship in semi-natural environments. Genes transferred with the transgene will include some which code for traits adapted to agricultural environments but inappropriate in the wild (e.g. pod shattering, low or inappropriately cued dormancy). This phenomenon (known as linkage drag) may explain why no crop-wild relative hybrid has become invasive in the UK. The few seriously invasive species have come from the 1 274 naturalised exotic species introduced in the UK usually for horticulture or by accident [see section 6.2, box 6.1 and a recent Nature report (Adam, 2003) mentioned by R. J. Berry on the Review website[24]]. Transgene stacking in sexually compatible wild relatives of GM crops, although possible, is likely to be rare for the reasons discussed above. However, if it did happen, it is theoretically

[24] http://www.gmsciencedebate.org.uk/topics/forum/0058.htm.

possible for the benefits of some combinations of transgenes to outweigh the disadvantages of linkage drag and result in wild plants with increased competitive ability. Therefore, risk assessments should, and do consider which transgenes are present in different GM varieties of the same crop and what the consequences of their combination in the same plant might be (for crop plants and any sexually compatible wild relatives). Assessing the consequences of some stacked traits on wild populations, whether they are from GM or non-GM varieties, is potentially difficult, although this may not be the case for other trait combinations (e.g. tolerance to different herbicides). The most direct way of assessing the fitness of any wild relative with stacked transgenes would be to create such plants and test their fitness under field conditions. However, given the current nature of the regulatory system in the UK, studies involving deliberately modified wild plants outside of contained conditions are very unlikely. There are few studies that have looked at the performance of hybrids of GM crops and wild relatives in semi-wild conditions (containing single traits) in the USA. Most of these data comes from agricultural weeds that have been deliberately crossed with transgenic crops (Snow *et al.* 2003; Spencer and Snow, 2001).

It is widely agreed that the spread of the transgene, and hence the consequences of gene flow, is likely to vary on a case-by-case basis. Genes conferring herbicide tolerance have the potential to create an agricultural weed problem if transferred to arable weeds (or volunteers). This can be seen from studies of oilseed rape in Canada where complete freedom among farmers to grow varieties tolerant to one of three herbicides (two of which were transgenic) has led to gene stacking and to multiple tolerance (Senior & Dale 1999, Hall *et al* 2000, Orson 2000, Beckie *et al* 2002 and Warwick *et al* 2003). Senior & Dale (2002) point out that careful management of herbicide tolerant crops can delay, or even prevent, the emergence of a herbicide-tolerant weed problem.

Plants containing herbicide tolerant genes are likely to survive and spread in conditions where the herbicide is being applied (in the last 40 years more than 120 plant species worldwide have developed herbicide resistant individuals under modern agricultural conditions). Elsewhere, and particularly in semi-natural environments such plants may be at a disadvantage compared to individuals without the herbicide-tolerance gene (Crawley *et al* 2001) and there is experimental evidence that herbicide-tolerance actually confers a cost on its possessor (Bergelsen *et al* 1996, Snow *et al* 1999). In other cases (e.g. resistance to insects conferred by the possession of the *Bt* gene) the transgene will not necessarily confer a cost under greenhouse conditions and will actually lead to increased fitness under insect pressure (Stewart *et al* 1997). It may also lead to increased seed production in semi-natural environments (Snow *et al* 2003). This reproductive advantage in the wild, an increase in fecundity, does not necessarily mean that the gene would increase the biological fitness of the plant (Bergelsen 1994, Snow *et al* 2003). In environments where the specific herbivores are absent (enemy-free environments) plants defended genetically against them may be out-competed by undefended plants (Agrawal *et al* 1999, Redman *et al* 2001). It is clear from studies of viruses in wild *Brassica* species in the UK that the complex interaction between different pathogens and different host species prevents generic assessments or predictions of the likely outcome of the transfer of a particular pathogen or herbivore resistance gene to a wild relative (e.g. Maskell *et al* 1999, Raybould *et al* 1999, Thurston *et al* 2001, Pallett *et al* 2002, Raybould *et al* 2003).

A contribution to the review has raised concern about crops genetically modified for male sterility and in particular the use of the *barnase* gene (Mariani *et al.* 1990 and 1992) in case it transfers to wild plant populations and causes their extinction (Mae-Wan Ho and Joe

Cummins)[25]. In transgenic plants the *barnase* gene is controlled by a promoter that restricts its expression to tapetal cells of the anther associated with the production of pollen. Plants containing the *barnase* gene will be male sterile and will need pollen from male fertile plants to reproduce - they cannot therefore transfer the gene to other plants (wild relatives or crop plants). The expression of a second gene, *barstar*, also controlled by a tapetum specific promoter in this same cell layer, stops the activity of the barnase gene product and restores male fertility. Consequently, plants containing *barnase* and *barstar* genes can produce pollen, which could potentially pollinate sexually compatible wild relatives as well as other crop plants (the likelihood of this occurring is discussed above and in section 6.2 respectively). However, in this case, approximately a quarter of the progeny of any crop/wild relative hybrid that does result will not be able produce pollen and therefore transfer the *barnase* gene to other plants. This is because *barnase* and *barstar* genes are not genetically linked in the GM crop (i.e. they are not in close proximity to each other on the same chromosome) and so will become separated in the progeny of future generations. Even if both *barnase* and *barstar* genes were transferred to a non-GM crop plant, or to a wild relative there would be no immediate consequences because seeds would be produced and oil harvested. The male sterile progeny from these plants would be greatly diluted by progeny from seeds from non-GM plants or wild-relatives that did not contain the *barnase* gene. The likelihood of *barnase* gene transfer is therefore extremely low, much lower than for most other genes. Many plant species have male sterility and this does not result in their extinction (reviewed by Williams, 1995). On the contrary, male sterility has been exploited in conventional plant breeding because it necessitates out-crossing and therefore generates genetic variation and hybrid vigour. However, in some crops male sterility may be associated with other characteristics that are not wanted, or it may not be stable. In such cases the use of genetic modification to confer male sterility may be useful. With respect to concerns about the characteristics of *barnase* gene expression, there is a body of quality evidence that shows that the barnase enzyme is restricting to specific cell layers and its activity is very effectively prevented when barstar is present (Mariani *et al*.1990 and 1992). If this were not the case, the GM crops in which this system is used would either not survive, or would perform poorly compared to their non-GM counterparts and there is no evidence of this.

Although the likelihood and consequences of gene flow must be assessed on a case-by-case basis and will differ in weedy and wild relatives, the evidence discussed above supports the view that some broad generalisations can be made based on our current understanding of population biology and genetics (and underpinned by the paradigms of evolutionary biology). Genes likely to confer fitness (e.g. virus resistance) have greater potential to lead to 'ecological release' (the expansion of a population locally following the removal or disablement of a regulatory mechanism such as herbivory or a pathogen) than genes which are neutral or disadvantageous (e.g. herbicide tolerance). However other constraints on population expansion such as density dependent competition could prevent an increase in population growth rates (discussed in Chapter 6.2). Overall, the fact that genes for pest and disease resistance inserted into crops by conventional breeding have not produced invasions of wild relatives in semi-natural habitats, coupled with the evidence that transgenes behave as naturally-occurring genes, suggest that predictions based on the tenets of invasion biology are supported by genetic evidence.

[25] http://www.gmsciencedebate.org.uk/topics/forum/pdf/0046.pdf

7.3.4 Is there general scientific agreement?

There is agreement that cross-pollination with wild relatives, where the latter are sexually compatible with the crop species, is likely to occur and the extent of hybridisation will vary from species to species and under different conditions. However as described above, the likelihood of gene flow depends not only on the range of factors influencing hybridisation but also on factors affecting the survival, growth and reproduction of the hybrid (introgression). The production of a crop/wild relative hybrid is but the first step in genetic exchange between populations.

The majority of scientific opinion argues that gene flow to wild relatives is not intrinsically harmful but that every transformation (each crop/gene combination), and every potential transgene/transgene conbination which could arise through gene stacking, should be examined on a case-by-case basis to assess whether gene flow may have harmful consequences to human health or the environment.

7.3.5 Are the issues unique to GM?

The phenomonen of gene flow from crops to weeds and from crops to wild relatives has been a part of agriculture for many hundreds of years, and remains a possibility in modern agriculture (see above). What is new is the possibility of introducing genes that code for entirely novel traits such as the production of novel enzymes or pharmaceutical products. This possibility provides the imperative for regulating GM technology on a case-by-case basis. There is currently no evidence to indicate that transgenes are more likely to transfer and persist in the wild than naturally-occurring genes.

7.3.6 Are there gaps in our knowledge or scientific uncertainties and are these important?

Although there are uncertainties about the scale and variability of crop to wild relative (and indeed crop to crop) gene flow, the major gaps are in understanding the potential consequences of gene flow. The effect of particular traits on the fitness of the weed or wild relative which may receive them is an important target of ongoing research (e.g. the studies of virus resistance in *Brassica* referred to above are a series of research projects funded under the BBSRC/NERC Initiative 'Gene Flow in plants and micro-organisms').

Ways are being sought of assessing the potential impact of transgenes on fitness that provide less expensive alternatives to the PROSAMO type experiments described in Chapter 6. These include targeted experiments, modelling and the development of protocols using a tiered approach (Linder 1999, Linder & Schmitt 1994, Bullock, Raybould *et al* 1999a, Hails 2000, Wilkinson *et al* 2003). All these approaches aim to assess the relative fitness of wild relative with and without the trait of interest (see section 6.2). If a range of GM varieties of the same crop are grown extensively in the UK, it is possible that transgene stacking will occur in sexually compatible wild relatives. Predicting the possible effects of potential transgene combinations in wild relatives (as well as in crop plants, see section 7.2) will become increasingly complex if a range of different GM crop varieties are grown on a commercial scale. This is unlikely to be a significant issue in terms the near future because of the number

and type of GM varieties that could be approved for commercial release in the UK. However, regulators will have to continue to be mindful of the consequences of gene stacking.

7.3.7 Likely future developments

Several technological solutions to containing or reducing gene flow from GM crops have been proposed (discussed in more detail in section 6.2). These can variously be labelled 'gene containment systems' and include insertion of the gene into the chloroplast rather than the nuclear genome (Daniell *et al* 1998), the use of chemical stimulants to express traits, through the action of an inducible promoter which prevent anther development or seed germination, and various mechanisms for preventing pollen production and dispersal (apomixis, cleistogamy). The risk assessment prior to growing crops expressing pharmaceutical or industrial proteins will need to have addressed the risk management issues around containment. Gene containment systems might be one genetic route amongst the considerations of physical, biological and genetic containment approaches.

7.3.8 Where there is important scientific uncertainty, what is the potential way forward?

The major uncertainties relate to the consequences of gene flow and must be dealt with on a case-by-case basis.

The way forward is the continuing assessment on a case-by-case basis of the consequences of gene flow to weedy relatives and those in semi-natural environments. As indicated earlier some of this research has been carried out or is underway. However ecological questions tend to lie along the critical path of any environmental risk assessment and some demand long-term research and/or monitoring.

7.4 CAN DNA FROM GM CROPS TRANSFER TO SOIL MICROBES?

In nature, how important and prevalent is horizontal gene transfer from plants to microbes in the soil, and does the presence of transgenic DNA make this more likely to occur? To what extent are the ecological effects of horizontal gene transfer from plants to soil microbes predictable?

7.4.1 Summary

Soil microbes are exposed to plant DNA from the normal processes of decay of plant material in soil. Most DNA is degraded, but there is a small but not zero possibility that genes in plant DNA will be acquired and expressed by soil microbes. However, the probability may be higher for transgenes in current use than for average plant genomic DNA because they contain DNA derived from bacteria. The chance of acquisition and expression by bacteria would be reduced by avoiding sequences of DNA that have similarity to bacterial DNA or that resemble bacterial insertion sequences or expression signals, and by using genes containing introns. Genes in chloroplasts may have an increased probability of being acquired and expressed because they are present in higher copy number and have bacterial-type signals. Ultimately, only acquisitions that are advantageous to the microbe have the potential to have ecological impact. Constructs that can rationally be predicted to cause harm if expressed in microbes must be avoided, but many constructs will be harmless because they will confer no advantage on microbes. In some cases, experimental tests may be required to confirm this.

7.4.2 Background

Horizontal gene transfer (HGT) means the transfer of genetic material between organisms with distant genetic relationships in such a way that the genes become heritable in the recipient. HGT is undoubtedly very infrequent, so it is hard to observe directly. Most evidence comes from events that happened long ago (detected by searching genomes for sequences that are shared between distantly-related organisms), or when the acquired genes are strongly beneficial to the recipient (as in the case of antibiotic resistance genes in disease-causing bacteria). While HGT undoubtedly occurs between bacterial species, the existence of HGT from higher organisms to bacteria is less well established. The issues raised here are parallel to those for the potential transfer to gut microbes (section 5.4).

HGT from plants to soil microbes was discussed at a GM science Review open meeting at the Royal Society of Edinburgh in January 2003. In addition, a number of contributions to the Review website are concerned with this issue. The points and questions raised fall into three broad categories (i) Whether and to what extent HGT occurs between GM plants and soil microbes (e.g. ISIS)[26] (ii) what the possible consequences might be (e.g. Brian Stratton[27]; Penny Hirsch[28]) and (iii) what the major uncertainties are (e.g. Greenpeace)[29]. The evidence, concerns and questions presented during the review have framed the writing of this paper.

[26] http://www.gmsciencedebate.org.uk/topics/forum/0030.htm
[27] http://www.gmsciencedebate.org.uk/topics/forum/0021.htm
[28] http://www.gmsciencedebate.org.uk/topics/forum/0085.htm

7.4.3 Range of views and quality of evidence

The frequency at which HGT occurs between plants and soil microbes.

Is the plant DNA available?

Yes. Plant roots slough off some dead cells, and all plant parts eventually die and decay in the soil. Most of the DNA is broken down in the dying cell, or digested by extracellular enzymes, or eaten by animals. However, some persists in the soil for months. There is no reason to suppose that the longevity of transgenic DNA is different from that of other plant DNA, but most studies have in fact looked at transgenic DNA because the issue has been raised in relation to GM plants. Studies have shown that transgenic DNA can be detected for at least four months (Widmer *et al.*1997, Hay *et al.* 2002) or up to two years (Gebhard and Smalla 1999). DNA was detected using the polymerase chain reaction (PCR), which is extremely sensitive and can detect just a few molecules of a gene, though it should be noted that soil often contains compounds that reduce the sensitivity of this assay.

In these experiments, and more generally, DNA may be protected from degradation by cell debris or by binding to clay in the soil, and this may affect its availability to bacteria (reviewed by Dröge et al. 1999). DNA adsorbed to sand or clay can transform competent bacteria (Lorenz and Wackernagel 1990; Chamier *et al.* 1993; Romanowski *et al.* 1993) and Lorenz *et al.* (1988) even showed increased transformation efficiency for *B. subtilis* as compared to transformation in solution. Others found lowered availability (Demanèche *et al* 2001a), especially for bound plasmid DNA (Chamier *et al.* 1993).

There are indications that pollen can be an accessible source of DNA in soil: a study by Meier and Wackernagel (2003) found the transgene in soil up to 50m from pollen-producing transgenic sugar beet plants, detected by both PCR and the transformation of *Pseudomonas stutzeri*.

If the transgene is located in the chloroplast genome, rather than the plant nuclear genome, then availability may be enhanced because, in green tissue, chloroplast genes may be thousands of times more abundant than nuclear genes. The relative availability of chloroplast and nuclear DNA has not been compared directly, but persistence of chloroplast DNA in soil has also been demonstrated (Ceccherini *et al.* 2003).

Can microbes take up plant DNA?

Yes. A significant number of bacteria have the ability take up DNA from the environment (they are "competent for transformation"). This competence is often induced temporarily and is sometimes confined to DNA from the same species, but uptake of foreign DNA is definitely possible in a number of species (Lorenz and Wackernagel 1994). Species that are not normally transformable can be forced to take up DNA in the laboratory by electric shock treatment (electroporation). It has been suggested that this might occur naturally through lightning, and this process has been simulated in the laboratory (Demanèche *et al.* 2001b), though probabilities may be low in the field.

The situation in fungi is less well studied than in bacteria, but laboratory methods for transformation have been developed in many species (e.g. Gietz and Woods 2001). Hoffmann

[29] http://www.gmsciencedebate.org.uk/topics/forum/0023.htm. This contains a link to Crops of Uncertain Nature, Controversies and Knowledge Gaps Concerning Genetically Modified Crops, An Inventory (A.J.C. de Visser, E.H. Nijhuis, J.D. van Elsas and T.A. Dueck).

et al. (1994) demonstrated transient expression of hygromycin resistance in the plant-pathogenic fungus *Aspergillus niger* infecting *Brassica* transgenic for the resistance gene, but obtained only a single stably resistant clone of the fungus. The mechanism of acquisition was unknown.

Can microbes incorporate transgenic plant DNA?

Acquired DNA may persist in a bacterial cell for some time and may even be transcribed and translated to make protein, but it will eventually be lost unless it is able to replicate. If it includes a plasmid origin of replication and can circularise, it may become established as an autonomous plasmid. Although transgenic constructs containing a plasmid origin have been made (Schlüter *et al.* 1995), this is not generally true. Normally, the DNA has to integrate into the genome, and the chances of this depend strongly on its sequence. Transposon terminal repeats would provide an obvious mechanism, but there is evidence that any stretch of DNA that has sequence homology to the bacterial genome can greatly enhance the rate of incorporation by homologous recombination (de Vries *et al.* 2001). Details vary between bacterial species, a perfect match between 26 base pairs in a row at one end of the incoming DNA is enough to allow recombination in *Escherichia coli*, whereas *Bacillus subtilis* requires a match at both ends, each of about 20 base pairs (Majewski and Cohan 1999). The stringency of this requirement may vary even within a bacterial species if mutations occur in the DNA repair systems (Vulic *et al.* 1997). Many transgenes are of bacterial origin, which would in principle increase the probability of finding homology in a recipient bacterial genome. However, it is common practice to modify the codon usage[30] of the gene in order to increase expression in the plant. It is likely that this will introduce more than one or two changes in each run of twenty bases, which will be enough to prevent homologous recombination in bacteria that possess the original gene sequence.

Can microbes express transgenic plant DNA?

The regulation of gene expression is different in the plant nucleus from that in bacteria, and the promoters that drive expression in plants may not work in bacteria, although some do. In fact, promoters vary among bacterial species, so it important to consider the likely recipients and a demonstration that a promoter is inactive in certain species should not be extrapolated to all bacteria. For example, a promoter that works well in *Rhizobium* may not function in *Escherichia coli* (Spaink *et al.* 1987). In any case, it is possible (with a correspondingly reduced probability) for the DNA to be inserted behind an existing promoter in the bacterial genome. Most plant nuclear genes contain introns that would not be correctly spliced out in bacteria, so no complete protein product would be produced. However, most transgenes in current use originate in bacteria and do not have introns, so effective expression in bacteria is more likely than for native plant genes. Genes from the chloroplast, whether native or transgene, are much more likely to be expressed in bacteria than are nuclear genes, because expression in chloroplasts is similar to that in bacteria, and most chloroplast genes lack introns. Alterations in codon usage made to improve expression of the gene in plants would be likely to reduce the expression in the original bacterial host, but bacteria vary greatly in their codon usage so some potential recipients might still be able to express the gene efficiently.

[30] this means altering the DNA code without changing the protein that it represents.

What direct evidence is there that bacteria can acquire DNA from plants?

There are no reports that natural soil bacteria have acquired genes in the field from crop plants, whether transgenic or not. However, there has been no systematic large-scale search for evidence.

There have been a number of laboratory studies seeking to determine whether it is feasible for bacteria to incorporate DNA from plant tissues. These studies have used conditions designed to maximise the chance of HGT occurring and of the result being detected. They have been reviewed in detail by Nielsen *et al.* (1998) and Bertolla and Simonet (1999). Citing this work, Nap *et al.* (2003) conclude that 'Several experimental studies have been published that all failed in demonstrating HGT from transgenic plants to bacteria'. However, the following evidence shows that such transfer is possible, albeit demonstrated in more or less artificial circumstances.

Bacteria can incorporate DNA from transgenic plants. Schlüter *et al.* (1995) studied the acquisition by the plant pathogen *Erwinia chrysanthemi* of a bacterial plasmid replication origin and marker gene that had been inserted into the genome of potato. They detected acquisition from purified plant DNA but not from plant tissue, and from a consideration of the various factors that they demonstrated to affect the rate they concluded that under field conditions HGT "is so rare as to be essentially irrelevant to any realistic assessment of the risk involved in release experiments involving transgenic plants".

Plant tissue can be a source of DNA for bacterial transformation. Gebhard and Smalla (1998) showed that a highly-transformable strain of *Acinetobacter* could acquire a kanamycin-resistance gene from homogenised leaves of transgenic sugar beet. However, the rate was low (10^{-10}) even though the potential for incorporation was strongly enhanced because the recipient bacteria had sequences that exactly matched part of the transgene (a "marker rescue" experiment).

Bacteria can acquire genes from plant tissue that has not been artificially prepared. Kay *et al.* (2002) repeatedly detected transfer of chloroplast-encoded sequences from tobacco to *Acinetobacter* in plants damaged by infection with the plant-pathogenic bacterium *Ralstonia*. Again, there was sequence identity between the incoming DNA and the recipient genome, which would strongly enhance the rate.

Evidence for HGT under field conditions has been sought but not found. Gebhard and Smalla (1999) showed that transgenes from sugar beet litter were detectable for up to two years in field soil. The transgenic construct included a bacterial kanamycin-resistance gene, but although kanamycin-resistant bacteria were abundant in the soil, this resistance was not caused by HGT from the beet because none of the 4000 resistant strains tested carried the transgenic DNA. The authors did detect the transgene by PCR in some samples of total DNA from mixed soil bacteria, but there was no evidence that this was derived from the bacteria rather than from unincorporated DNA.

There is one scenario that does not strictly involve gene transfer from plants to bacteria, but has given rise to some concern. The bacterium *Agrobacterium* is commonly used to transfer genes into plant cells because it possesses a natural mechanism for this. After transfer, antibiotics are applied to remove the residual donor bacteria. However, Barrett *et al.* (1997) showed that the commonly-used levels of antibiotics were insufficient to kill all the bacterial cells, so that bacterial contamination persisted over several months. Since the transgenes are

normally carried on a transmissible plasmid in the *Agrobacterium*, there would be a substantial probability that they would be transferred to soil bacteria if such an infected plant were planted out. This could be an issue during the early stages of GM crop breeding, though it would not be likely to affect commercial seed since this would be several plant generations removed from the initial transformation, and *Agrobacterium* is not seed-transmitted. For vegetative crops (e.g. potatoes), this situation is considered in risk assessments.

What do genome sequences reveal about HGT?

Now that the genomes of many bacteria and quite a few higher organisms have been completely sequenced, it is possible to examine them directly for genes that show a pattern of evolutionary relationships which is clearly different from that supported by the majority of genes. Many such examples have been identified, including genes that appear to have transferred between higher organisms and bacteria, but the transfers would have happened long ago, often hundreds of millions years ago (Koonin *et al.* 2001, Brown 2003). Such ancient events are not relevant to the issue of HGT in relation to GM crops.

There is, so far, no evidence for recent successful establishment of plant genes in bacterial genomes. Examination of the complete genomes of an *Agrobacterium* and three rhizobia, all soil bacteria that are very closely associated with plants, provides no evidence of any genes that are very similar to plant genes (Kaneko *et al.* 2000, Galibert *et al.* 2001, Wood *et al.* 2001, Kaneko *et al.* 2002).

What these bacterial genomes do reveal is abundant evidence that some genes have been transferred between bacterial species. While transformation is a possible mechanism here, many bacteria carry conjugative plasmids or transposons that provide a more robust means for genes to spread within and between species. The significance of these processes in the environment has been reviewed many times (e.g. Dröge *et al.* 1999, Davison 1999, van Elsas *et al.* 2003). The surface of roots provides a favourable environment for such transfer because of the availability of nutrients, high bacterial densities which trigger conjugation through quorum sensing[31] (e.g. Oger and Farrand 2002), and possible activating compounds in plant exudates (Zhang *et al.* 1993). Transformation can also be enhanced by compounds exuded by plant roots (Nielsen and van Elsas, 2001). Experimental evidence for the spread of genes between bacteria in the soil environment has been reviewed by Bailey *et al.* (2001)[32]. The relevance of this for the issue of HGT from plants is that, if plant-derived genes were to get into some component of the bacterial community that may be particularly prone to transformation, there is a likelihood that they could be transferred to other bacteria that are not themselves readily transformed.

Predicting the consequences of HGT from plants to bacteria

Any potential consequences of HGT depend on the fate of the recipient microbe. A single microbial cell is too small to have any detectable environmental impact, even if it carries a potent transgene, so the critical question is whether this cell will multiply into a sizable population. It will only do this if it confers some advantage, or at the very least, does not confer any disadvantage over the other microbes that it is competing with.

[31] Quorum sensing is a phenomenon by which some bacteria measure their own abundance - it involves the release and detection of signalling molecules.
[32] http://www.defra.gov.uk/environment/gm/research/pdf/gm_research_17.pdf

To address the potential environmental impact of HGT it is therefore important to ask two questions. Firstly whether expression of the gene would benefit the recipient, because if it is disadvantageous then the number of cells will remain too low to have a detectable effect. Secondly whether the spread of microbes carrying transgenes would alter the functioning of the ecosystem in any significant way. It is obvious from these considerations that the potential consequences of HGT depend on the exact nature of the transgenic DNA, and there can be no general answer.

Many of the relevant issues are addressed in some detail in a report for Defra authored by Bailey *et al.* (2001). This report is concerned with the possibility and potential consequences of HGT from GM bacteria, but of course the subsequent fate of the recipient will be subject to similar considerations regardless of the source of the transgenic DNA.

There is a considerable body of theory concerning the conditions under which a type of organism that is initially rare will establish and spread. A selective advantage is important, but in the early stages there is a high probability that the new type will die out through chance events even if it has an advantage. Although these generalisations are undoubtedly true of the products of HGT, the theory can offer few quantitative predictions without specific knowledge of the relevant parameters in a particular case.

There have also been many experimental studies on the spread of plasmids, etc., through bacterial populations, and the results are concordant with theoretical predictions. However, these have been simple laboratory systems such as well-mixed liquid cultures or plain surfaces (e.g. Simonsen, 1990), which are not representative of the spatial complexity and heterogeneity of the soil and plant environment. There is no immediate prospect of a quantitative predictive theory for the population dynamics of individual microbes in soil, but the same is true for many other complex systems that nevertheless have predictable average properties.

In a similar vein, comparative genomics has revealed that all natural genomes are full of 'accidental' features, such as transposable elements and genetic rearrangements that differ from one species, and even individual, to another (for examples of soil bacterial genomes, see Kaneko *et al.* 2000, Galibert *et al.* 2001, Wood *et al.* 2001, Kaneko *et al.* 2002). Bacteria, in particular, have a large fraction of 'accessory DNA' that varies in content from strain to strain and is often subject to HGT. In this context, there is clearly no longer any basis for the view that the genome is a finely-tuned machine that might be disrupted by the introduction of a 'foreign' gene, causing the organism to 'run amok' in some unpredictable way. Bacterial genomes are, in general, resilient to the acquisition of new genes, so the focus has to be on the specific effect of the particular gene.

Even if the transgene confers an advantage that allows its recipient to increase in frequency, a key issue is whether overall soil functioning is affected – this is the case when assessing any change e.g. as a result of pesticide usage or altered crop rotation. Soils are dynamic systems that are in constant state of flux, for example they are affected by the weather, agrochemicals, what crop and even what variety is grown (reviewed by ACRE's soil ecology sub-group)[33]. Against this background, the significance (if there is any), of most change is not apparent. The scientific evidence shows that change is often reversible and soil functioning is robust. For

[33] http://www.defra.gov.uk/environment/acre/soilecology/acre_soilecology_interim.pdf

example, Griffiths *et al*. (2001) created soil communities with biodiversity reduced to a half, but found no change in measures of overall functioning.

One issue that has attracted a good deal of attention is the potential transfer of antibiotic resistance genes from GM crops to bacteria, and the fear that this may lead to increased resistance in bacteria of clinical importance. Antibiotics are a normal feature of the soil ecosystem. Most natural antibiotics were isolated from soil microbes (bacteria or fungi), and antibiotic resistance genes originate from these same organisms. Of course, it was the spread of these genes into clinical bacteria that first alerted microbiologists to the potential of HGT between different bacterial species. This spread has been driven by strong selection imposed by the clinical use of antibiotics and their widespread use as growth promoters in animal husbandry. Antibiotic resistance genes have been used in the creation of GM plants because they provide an effective means to select the transformed cells. They are not a necessary component of the final product and, as effective alternative methods are developed, it is likely that future GM plants will not carry antibiotic resistance genes. Nevertheless, it is improbable that GM plants would significantly affect the incidence of clinical antibiotic resistance through transfer in the soil, for two reasons. Firstly, the resistance genes in question are already widespread in bacterial populations, including those in clinical settings, so it is much more likely that a bacterium will acquire them from another bacterium (HGT between bacteria being relatively common) than from a plant (HGT being extremely rare). Secondly, the concentration of man-made antibiotics in soils is very low compared with clinical usage, so there will not be a similar level of selection favouring a bacterium that receives the resistance gene in the soil. Set against the first argument, it must be acknowledged that the commercial growing of a GM crop would provide an enormous multiplication in the number of copies of the gene that might offset the very low HGT rate, but this is irrelevant because the dynamics of antibiotic resistance spread are driven by the strong selection pressures rather than by the rate of HGT (which is relatively rare even in clinical settings).

This emphasis on selection pressure is key to assessing the potential fate of a transgene if it were to transfer to a microbe. The question that needs to be asked is whether a particular gene could confer a benefit on its recipient. There are some plausible cases in which the answer might be positive. To take a hypothetical example, the herbicide glyphosate is also toxic to some fungi (Morjan *et al.* 2002), so glyphosate resistance would confer an advantage on a fungus in the presence of the herbicide. If the fungus were able to acquire the resistance gene from a GM crop, then there is a possibility that the fungus with the transgene would spread within the herbicide-treated environment at the expense of other fungi. Whether this was of any ecological or agronomic significance would depend on the nature of the fungus. If the only phenotypic effect of the gene was to confer herbicide resistance, then the transgenic fungus would not be distinguishable from its non transgenic relatives except in the presence of the herbicide, so it should have no impact outside the crop. This hypothetical scenario illustrates the kind of questions that need to be asked when assessing the potential consequences of gene flow from GM crops, and more generally in assessing the impact of organisms with new traits (in particular, see sections 6.2 and 7.3).

7.4.4 Is there general scientific agreement?

There is good evidence and general agreement on the following points:

- DNA from crop residues remains available in the soil for months.

- The chloroplast genome is present in higher copy number than the nuclear genome in plant material.
- Some bacteria can acquire DNA by transformation.
- The probability that acquired DNA will be incorporated into a genome is greatly enhanced if it includes sequences closely similar to sequences in the recipient's genome.
- The probability that acquired genes will be expressed is enhanced if they resemble bacterial genes in their control elements, their codon usage, and in lacking introns.
- The transfer of genes from GM plants to soil bacteria under field conditions has not yet been observed.

From the available evidence, some authors have concluded that the rate of HGT from plants to microbes is so low (perhaps zero) that it can be neglected for the purposes of risk assessment. However, all the necessary stages of the process have been demonstrated individually, so it would be prudent to assume that they can occur, albeit at a rate that is too low to have been detected yet. Enormous areas are covered by crops (maize is currently grown on 140 million hectares worldwide, and there may be 10^{16} bacteria per hectare of soil), so even very low rates might not be negligible.

The critical question is whether the transgene would confer a selective advantage on a microbial recipient in the particular environment in which it is living, because if it does then even a very rare HGT event could lead to a significant effect. This has to be assessed on a case-by-case basis for each transgene and the context in which it will be used, considering all plausible classes of recipient.

7.4.5. Is the issue unique to GM?

As all plant roots slough off dead cells and plant parts eventually die and decay, soil microbes are exposed to significant amounts of native plant DNA. The consequences if gene flow were to occur, are no more predictable for native plant DNA given the complex, diverse and fluctuating nature of soil ecosystems. There is no evidence from genome analysis for the acquisition of normal plant genes by soil bacteria. However, one might predict that HGT from transgenic crops will be more likely if the DNA contains sequence that is homologous to genetic material in the soil microbes.

Agricultural practices such as ploughing, fertilisation, irrigation and the growing of monocultures have large effects on the size and composition of soil microbial populations. The fact that soil processes continue under these circumstances, although rates may be altered, is evidence of the robustness of soil functional communities. Any additional perturbation caused by the introduction of a gene has to be considered against this background.

7.4.6 Are there gaps in our knowledge or scientific uncertainties and are these important?

Little is known about the proportion of bacteria in a given community that are naturally transformable, i.e. competent to take up DNA (Dröge *et al*. 1999). The competence of bacteria to take up DNA in natural environments is poorly understood. However, if the assumption is that the potential rate of HGT from plants to certain bacteria is not zero, and we know that

genes can spread between bacteria, then policy is not dependent on a more precise estimate of the rates.

HGT to other microbes, e.g. fungi and protists, has not been as well researched as for bacteria. Again, there is some indication that the rate may not be zero. Since these are eukaryotes, some further consideration should be given to the likelihood of incorporation and expression of the transgenic DNA used in GM plants, as the work directed at bacteria will not be applicable.

In many cases the obvious effect of a transgene in a potential microbial (or, at least, bacterial) recipient is easily predicted. An antibiotic resistance gene will confer antibiotic resistance, and so on. However, a gene may also affect other cellular processes, which could be revealed by transcriptomics, proteomics or metabolomics. Some representative studies of this kind would establish whether this is an issue that needs further attention.

Even if we know how a genetic change affects an individual cell, our understanding of soil ecosystems is insufficient to predict whether a gene will afford an advantage and, if so, what environmental impact(s) it will have. Over the last decades, microbial ecology has taught us that microbial populations can vary rapidly over time and space, so that only really major effects will be distinguishable against the natural fluctuations. However, ecosystem functioning appears to be quite resilient to changes in individual microbial components. A better understanding of microbial ecology is clearly desirable for all kinds of reasons, and will increase our confidence in assessing the potential consequences of all kinds of perturbations. What determines the establishment and spread of new genotypes is particularly relevant. At a more specific level, the experimental introduction of potential transgene constructs into representative bacteria may be necessary in some cases where the possible effects on the fitness of the recipient cannot be predicted with reasonable certainty.

7.4.7 Likely future developments

GM plants that have transgenes in plastids rather than the nuclear genome are being developed. This may be useful to reduce gene transfer through pollen or for achieving higher expression levels. However, transformation of plastid genomes (i.e. chloroplasts) may facilitate HGT to bacteria because of the increased copy number and closer relationship to prokaryotic gene structure.

Conversely, antibiotic resistance genes are being phased out and should eventually cease to be an issue.

Most current transgene constructs are based on ‘natural’ components, particularly bacterial genes, and it can be argued that they are unlikely to confer significant benefits on bacteria that have been exposed to them already by HGT from other bacteria. If constructs become increasingly ‘novel’, with substantial synthetic sequences, then this argument will have less force and the effect of the constructs may need to be explicitly tested in representative bacteria. On the other hand, the potential for homologous recombination will be reduced.

7.4.8 Where there is important scientific uncertainty what is the potential way forward?

Research

We need more knowledge and understanding of soil ecosystems. As a start, it would be useful to define methods to measure a set of meaningful parameters of ecosystem state and function and to collect baseline measurements against which the effects of treatments can be assessed. ACRE have established a soil ecology sub group to consider the potential generic effects that GM plants and the agronomic practices associated with them might have on soil ecosystems and how these might be measured. This requires an understanding of the changes
that occur in soil ecosystems associated with the cultivation of non-GM crops in order that changes associated with the release of GM crops can be put into context and parameters that it would be meaningful to monitor can be identified. In March 2003, the sub group produced an interim report that reviews the current state of knowledge of soil ecosystems relevant to the potential impacts of GM plants[34].

In the case of transgene constructs whose effect in bacteria is not readily predicted, there may be a need to test directly in representative potential recipients.

Technological approaches

The potential for transfer and expression of transgenes from GM plants to soil bacteria might be minimised by removing unnecessary vector DNA that provides homology with soil microbes (in particular origins of replication and sites for transposition) and introducing introns where possible (e.g. Libiakova *et al.* 2001). This precautionary approach is in line with ACRE's guidance on best practice for designing future GM plants.[35]

Regulatory approach

Given that we cannot guarantee that the probability of gene transfer will ever be truly zero, careful consideration should be given to the likely consequences of transgene expression in any plausible microbial recipient, and transgenes should be avoided if there is a reasonable expectation of harm if they were to get into the wrong organism.

When ACRE assesses the safety of the deliberate release of GMOs into the environment, this includes their potential impact on soil ecosystems. These risk assessments are conducted on a case-by-case basis and take into consideration direct, indirect, immediate and delayed effects principally associated with expression of the transgene(s) inserted to create the GMO. ACRE takes a precautionary approach and assumes that HGT from plants to soil microbes will occur and considers the potential consequences on a case by case basis. It is the view of this panel that this is the most effective way of considering HGT from GM crops to soil microbes.

[34] http://www.defra.gov.uk/environment/acre/soilecology/acre_soilecology_interim.pdf.
[35] http://www.defra.gov.uk/environment/acre/bestprac/guidance/index.htm

7.5 CAN GENETIC MATERIAL IN GM PLANTS TRANSFER TO VIRUSES?

Can plant-virus-derived transgenes recombine with, and be transferred to viruses? If horizontal gene transfer is possible between GM plants and viruses could this result in new viruses that could cause irrecoverable damage to the ecosystem or to crops?

7.5.1 Summary

Since 1986, many thousands of transgenic plant lines expressing one or more functional or dysfunctional viral sequence(s) have been shown to render the GM plant resistant or even immune to subsequent virus inoculation. The technical facility, durability, efficacy and heritability of this approach are now well-established.

In the past, any weakness in, or complete absence of 'natural' resistance genes in the breeding stock of many virus-susceptible non-GM crops required the liberal use of pesticides to control the insect, fungus or nematode which naturally transmitted the devastating virus. A specific virus-targeted resistance transgene in a GM crop variety thus can offer a selective, traceable and environmentally sustainable route to protect crop yield and quality.

In addition to the many thousands of contained laboratory, glasshouse and small-scale field trials, several GM crops (e.g. yellow crookneck squash, sweet potato and papaya) that express viral sequences, which confer functional field resistance to devastating wild-type viruses have been grown commercially on large-scales, over the past 7 years. No new types of virus have been reported in association with the development, or commercialisation of these crops. One large-scale field study has been published that looked specifically for evidence of altered properties in infecting viruses and recombination (HGT) between infecting viruses and GM plants containing virus-derived transgenes over a six year period, none was found (Thomas *et al.* 1998).

Many artificial, laboratory-based recombination-selection systems can and have been established between two or more debilitated (mutated) viruses in A non-GM host plant, or between a more-or-less defective virus in a GM plant containing a transgene sequence capable of restoring wild-type virus. In most cases, usually depending on the strength of the evolutionary selection pressure applied, wild-type virus can be recovered in some plants (from <1% to approx. 30%) through homologous recombination (template strand switching) and selection during RNA-RNA replication (as in most plant viruses). Similar laboratory results have been reported during RNA-DNA reverse transcription (in plant Pararetroviruses) or through DNA-DNA replication (in Geminiviruses). In contrast, in nature, infecting viruses will be fully viable wild-type strains and mixed infections are common, providing far greater opportunities for virus-to-virus genetic reassortments, recombination, etc.

The seminal paper on this topic (Greene and Allison, 1994) decribes how a deletion mutant of cowpea chlorotic mottle virus (CCMV) was restored to wild-type in 3% of defective CCMV-inoculated GM plants. Homologous recombination had occurred between the debilitated virus and a transgene transcript that had a perfect sequence overlap of 338 nucleotides and a fully functional 3'-replication origin. When the replication origin was later removed the frequency of recombination fell dramatically (Greene and Allison, 1996).

Recombination is well-documented and plays a key role in natural virus evolution (see Tepfer, 2002). As yet, however, there is no evidence of such recombination (i.e. horizontal viral transgene transfer; HVTT) occurring in the field where single transgene resistance has been engineered against a wild-type virus. The possibility that HVTT might occur and its possible virological, biological and/or ecological consequences have been broadly discussed and speculated on in virtually every review article on this subject since the earliest days of the technology. The nature of any hazard, the probability of its occurrence and any possible consequences, or lack thereof, remain real but manageable issues for those who design, produce and test virus-resistant GM crops.

It is theoretically possible, but without precedent, that any tested and approved viral transgene sequence could, or would render any invading wild-type virus more pathogenic, affect its transmissibility, pathogenicity or other characteristics. On the contrary, high mutation frequencies, genome reassortments and recombination events in natural (often mixed) virus infected plants are common. Hence, any new genetic trait which is beneficial to a virus is presumed to have been selected already, through millenia of evolution, especially in the highly mutable pool of genomes which comprise the "quasi-species" of each RNA virus (>90% of all plant viruses). Indeed, since the 1970s, the accepted and approved practice of intentionally infecting many valuable crops (e.g. glasshouse tomatoes) with a mild strain of a virus to "cross-protect" them from infection by severe, devastating strains of the same or a related virus poses far greater (and documented) opportunities for recombination to create new virus strains (e.g. citrus tristeza virus in Brazilian oranges – see section 7.5.5).

The recommendations contained in an earlier (1999) DETR Research Report remain relevant today, although with our improved knowledge and understanding of RNA-interference (RNAi) and gene silencing/plant defence pathways, many of the risk issues that were proposed last century can now be avoided when designing new viral resistance transgene strategies.

Containment of any newly emerging plant virus is achieved through standard current and widely accepted phytosanitary control measures including replanting with healthy stock, spraying with pesticides or heat or chemical soil sterilisation to limit virus spread by killing its insect, fungus or nematode vector.

7.5.2 Introduction

Most first-generation GM plants (post 1983), whether made and used for research or commerce, contained short DNA sequences derived from plant viruses. These included non-coding elements used to regulate expression of any novel, functional transgene. Popular early examples were the so-called 35S and 19S promoter and terminator signals from the common Caulimoviruses (e.g. cauliflower mosaic virus, CaMV; or figwort mosaic virus, FMV – see later discussion on: *what effects could interactions between viruses and transgenes have*?). These sequences respectively start or stop transcription, the process by which the natural viral or transgene double-stranded DNA template is copied into a single-stranded messenger RNA (mRNA) for subsequent translation into a protein (the length/size of the viral RNA made dictates the naming of the promoter and terminator). The 35S promoters also generate the long viral RNA template used for viral reverse transcription (copying back) into daughter DNA molecules – a process peculiar to Caulimoviruses and other so-called plant Pararetroviruses. Still other plant virus-derived sequences have been used, generically, to

increase expression of a novel protein from a transgene. These include several short, non-coding viral mRNA leader sequences (typically only 10-100 nucleotides long) that recruit the protein synthesising machinery of the cell extremely efficiently.

In other cases, commencing in 1986, GM plants were created that contained a DNA copy of a whole plant viral gene or a non-functional fragment of a viral gene. Such plants were found (somewhat serendipitously at first) to be resistant to subsequent challenge infection by the same or a closely related plant virus. Some of the first field trials with GM plants (1986-1990) included those that expressed a functional viral coat protein gene that conferred resistance to challenge virus infection. RNA plant viruses represent the vast majority of plant viruses and hence have been targets for almost all virus-derived pathogen resistance transgene strategies in GM plants over 17 years. Indeed, reports of successful transgene-mediated resistance against DNA plant viruses are relatively rare. Thus while recombination-selection and rescue events through RNA-DNA reverse transcription (Pararetroviruses), or DNA-DNA replication (Geminiviruses) are less dependent on the presence of a viral replication origin (cf RNA viruses), any GM-based field resistance strategy may offer limited success with these virus types anyway. This may be because RNA viruses are more susceptible to transgene-derived RNAi-mediated cellular silencing at an early stage of infection (discussed later).

The issue of whether or not viral transgene DNA (or RNA copies thereof) could recombine with naturally occurring viruses in GM crops, and the possible consequences of such events were raised in the GM Science Review by Econexus (JR Latham & RA Steinbrecher, Royal Society of Edinburgh Meeting 27 January 2003[36]).

The same issues have been speculated-on and reviewed extensively over many years revealing a broad diversity of opinion (see De Zoeten 1991; Gibbs 1994; Falk and Bruening 1994; Allison *et al*. 1996; Miller *et al.* 1997; DETR, 1999; Hammond *et al*. 1999; Rubio *et al.* 1999; Tepfer 2002 and many references contained therein).

More than 1000 plant viruses have been described and studied in greater or lesser detail over the last 100 years. Collectively, they are ubiquitous in nature and affect all plants, including all food crops, and even trees. Individually, however, their host-range may include only one or a few species, or up to 400 different species of plant. As omnivores or vegetarians, we consume plant viruses constantly without any ill-effects. They multiply (replicate) efficiently inside living susceptible plant cells, sometimes only in specific cell types, and usually accumulate to very high copy numbers (over 100,000 virus particles per cell, is typical). Almost all plant viruses consist of a geometrically assembled shell of coat protein subunits (the capsid) that protects the delicate (esp. RNA) and relatively small genome of the virus. All plant viruses encode three or more proteins. Most plant viruses (92%) use single-stranded RNA as their genetic material. Of these, over three-quarters use positive-sense RNA [i.e. as with cellular mRNA, the packaged ("encapsidated") viral RNA uses the cellular machinery to code directly for one or more proteins]. The first complete viral genome sequence (tobacco mosaic virus) comprising 6395 nucleotides of single-stranded RNA was published in 1982. Minor taxa (groups) of plant viruses have double-stranded RNA, or single- or double-stranded DNA as their genetic material inside virus particles of various shapes. Plant viral satellite RNAs, viroids and virusoids also exist in nature, but are not considered further here.

[36] Abstract: http://www.gmsciencedebate.org.uk/meetings/pdf/270103-speaker-2.pdf
Transcript: http://www.gmsciencedebate.org.uk/meetings/pdf/270103-transcript.pdf

A complex series of spatial and temporal interactions between virus-encoded proteins and RNA or DNA genome sequences, and host proteins and sub-cellular structures is required for successful viral multiplication (replication). GM plants that express a functional or dysfunctional plant viral sequence at the wrong time, wrong place or in the wrong amount, can interfere with one or more of these delicate stages in the normal virus infection cycle and thus render the plant phenotypically 'resistant'. Resistance may be complete or partial, and directly attributable to the functional or dysfunctional viral protein being expressed, or to activation and virus-targeting of an intrinsic plant cell pathway responsible for post-transcriptional gene silencing (PTGS) through highly sequence-specific RNA degradation.

Global estimates of crop losses due to all viruses range from 5-20% but can be 100%, locally, especially in sub-tropical and tropical regions in developing countries where there are high numbers of insects that transmit viruses to commercial and subsistence crops; and where resource-poor farmers cannot afford effective pesticides. As well as insects, fungi and nematodes transmit specific viruses between plants through feeding and/or wounding, which allows plant viruses to enter and infect susceptible plant cells. Most agriculture in industrial countries relies on traditional (enhanced) breeding and selection to deploy natural resistance to viruses (where available). This is backed-up by strict phytosanitary (plant quarantine) controls, high health status planting material, and/or extensive use of agrochemicals and soil-sterilisation techniques to control the insect, fungal or nematode vectors which spread viruses in nature. It is neither technically nor economically feasible to spray antiviral chemicals against plant viruses. Mechanical transmission (through handling, pruning etc) or vegetative transmission (through tubers, cuttings etc.) are alternative means of spread. Very few plant viruses are seed transmitted, and even fewer are pollen-transmitted. Plant viruses are ubiquitous and a natural part of our diet. They cause no disease or harm to herbivores. Some plant virus particles are so robust they pass through the gastro-intestinal tract intact. There is no evidence of any HGT between plant viruses and humans – despite eons of co-existence with our raw food crops.

As with all pathogens (and pests), any large-scale deployment of a new crop exhibiting single dominant genetic resistance (GM or non-GM) creates a selection pressure that will favour the emergence of resistance-breaking strains of the virus, fungus, bacterium, or pest. The rapidity with which this occurs will vary, case-by-case. With their short replication cycle and high copy numbers, viruses naturally evolve and recombine rapidly. Viruses that multiply (replicate) only by copying RNA-into-RNA have no molecular mechanism to repair spontaneous genetic errors (point mutations) that occur about once in every 1000 nucleotides. Hence, a typical plant virus with a genome of 6000 nucleotides will carry 6 random point mutations. This led to the "quasi-species" hypothesis for RNA virus populations, in which the best adapted "type" strain predominates; but infinite permutations arise naturally and continuously, to be selected under altered conditions. In practice it is remarkable how stable the "type sequence" remains (e.g. when the original TMV isolate made in 1935 was compared with the modern type strain of TMV that has gone through infinite replication cycles).

Many hundreds of examples of enhanced resistance against one or more viruses in GM plants using a wide range of virus-derived transgene sequences in a range of crop and model species have been published and reviewed (see 392 references cited in DETR, 1999; Wilson 1993). Much positive interest has been driven by the promise of a limitless supply of single dominant resistance genes which can be introduced simply, rapidly and durably into existing elite germplasm without loss of desirable agronomic or quality traits and without prolonged back-crossing programmes. Moreover, for many viruses and crops, native single dominant

resistance genes either do not exist or have not been identified in sexually compatible germplasm for breeding.

There are several examples of exploitation of virus-derived transgenic resistance to viruses. Although the number of crop varieties that are already in commercial cultivation is not high, there are many more reports of small-scale experimental tests of different crops with transgenic resistance to viruses (see Box 7.1).

Box 7.1

An example of successful field control of a devastating virus is given by commercial-scale cultivation of genetically modified **papayas** (transformed with the coat protein gene of ***papaya ringspot virus***) under high disease pressure conditions in Hawaii (Souza *et al.* 1999; Ferreira. *et al*. 2002: Gonsalves *et al.* 2002)

Trials of **wheat** varieties transformed with the coat protein gene of ***wheat streak mosaic virus,*** which showed some resistance to WSMV in glasshouse experiments (Sivamani *et al*. 2000 and 2002), showed that incorporation of the replicase or coat protein gene from WSMV did not provide field resistance to viral infection. In general, transgenic lines yielded less than their parent cultivar, 'Hi-Line', although resistance to WSMV was shown in glasshouse tests (Sharp *et al.* 2002).

Stable, heritable resistance to **rice yellow mottle virus** (RYMV) was reported in rice varieties transformed with a transgene encoding the *RYMV replicase* gene. In the most extreme cases, there was complete suppression of virus replication (Pinto *et al.* 1999).

Transformation of commercial **potato** cultivars with replicase (Thomas *et al*. 2000) and coat protein (Murray *et al.* 2002) genes of ***potato leaf roll virus*** (PLRV) resulted in a high degree of resistance to PLRV in some lines, although the results of field trials were less impressive than the outcome of glasshouse tests. Also, transgenic potatoes have been made with some resistance to the devastating, severe ***potato virus Y***–NTN isolate (transformed with the coat protein gene of PV-NTN) (Racman *et al.* 2001) and to Potato virus X (transformed with PVX coat protein) (Doreste *et al.*2002).

Field trials of **chilli pepper** transformed with ***cucumber mosaic virus*** and ***tobacco mosaic virus*** sequences showed milder disease symptoms and increased yield (Cai *et al.* 2003).

Tomato plants transformed with a fragment of the replicase gene of ***cucumber mosaic virus*** (CMV) showed resistance to CMV, and the selected lines are being used as breeding material for CMV resistance (Nunome *et al.* 2002). Transgenic **soybean** plants resistant to ***soybean mosaic virus*** **(**SMV) were obtained by transforming with the coat protein gene and 3'-UTR. Lines highly resistant to SMV were selected (Wang *et al*. 2002).

Resistance to ***carnation mottle virus*** (CarMV) was observed in **carnation** plants transformed with the CarMV coat protein gene (Yu *et al.* 2002). Heritable transgenic resistance of **sweet potato** plants to ***sweet potato feathery mottle virus*** (SPFMV-S) was conferred by transformation with SPFMV-S coat protein gene (Okada *et al.*2002).

Transgenic **Mexican lime** trees resistant to ***citrus tristeza virus*** (CTV) were generated by transformation with the CTV coat protein gene. Protection was also efficient against non-homologous CTV strains and was generally accompanied by high accumulation of CP in the protected lines, which suggest a protein-mediated CP-mediated protection mechanism (Dominguez *et al.* 2002).

The list of transgenic plants with functional resistance to viruses is likely to be extended as work continues in many laboratories (several hundred) worldwide. For further examples, see materials selected from just one recent meeting; the June ***2002 Congress on In Vitro Biology, Orlando, USA*** (**see Box 7.2**).

Box 7.2: Transgenic resistance to viruses in a variety of crops was reported recently: 2002 Congress on In Vitro Biology, Orlando, FL, USA, June 25- -29.

Kamo K., Gera A., Cohen J. and Hammond, J. Transformation of **Gladiolus** for resistance to **bean yellow mosaic virus**.

Hily,J.M., Malinowki, T., Ravelonandro, M.and Scorza R. Post-transcriptional gene silencing (PTGS) results in **PPV** resistance of transgenic **plum** trees after four seasons of growth in the field.

Hanbing An., Sarita E.V, Verchot-Lubicz, J. Transgenic resistance in **wheat** containing **soilborne wheat mosaic virus** (SBWMV) genes.

Oropeza M., Abouzid A. M., Miller J. D., Comstock J. C., Gilbert R. A. and Gallo-Meagher M. Analysis of transgenic **sugarcane** plants containing an untranslatable **sugarcane mosaic virus strain E** coat protein gene.

Scorza R., Ravelonandro M., Callahan A. M., Malinowski T., Damsteegt V. D., Levy L. and Briard P. Studies of **plum pox virus** resistance in transgenic **plum** C5 and its progeny.

Reustle G. M., Wetzel T., Jardak R., Ebel R., Worl R., Meunier L., Becker M. and Krczal G. Genetic engineering of **grapevin**e rootstocks to induce **nepovirus** resistance.

Raquel H., Lourenco T., Batista, R. and Oliveira M. M. **Almond** (Prunus dulcis Mill.): Preparing and testing of constructs for resistance to **prune dwarf virus** (PDV).

7.5.3 Range of views and quality of evidence

A survey of 23 UK and 49 overseas public and private sector organisations actively involved in GM crop science and virology in 1995-96 provided an analysis of the gene sequences, viruses and target plants being used (DETR, 1999). Most respondents cited RNA-RNA recombination or transcapsidation (i.e. the packaging of the genome of an invading virus in the coat protein of another virus expressed as a transgene) as potential hazards, although many saw no additional hazard beyond what would happen in nature during mixed infections by endemic viruses [up to 11 different viruses have been reported in a single plant (Falk and Bruening, 1994)]. Interestingly, those who had carried out field tests and had real data perceived fewer, if any, hazards than those preparing to do so or speculating on possible events.

However, one minority viewpoint expressed at the open meeting at the Royal Society of Edinburgh[37] contends that:

(i) genetic material from GM crops containing DNA derived from viruses will inevitably recombine with and transfer to naturally occurring viruses that infect them and that this could result in new virus strains which, in the worst case scenario, could cause irrecoverable ecosystem or crop damage.

(ii) the probability of HGT of transgenes is greater than for mixed infections because transgenic crops may result in new or enhanced opportunities for virus recombination (De Zoeten 1991; Gibbs 1994; Allison *et al.* 1996) – even though there is no evidence for this.

[37] Abstract: http://www.gmsciencedebate.org.uk/meetings/pdf/270103-speaker-2.pdf

(iii) our understanding of virulence determinants and ecological fitness is not sufficient to predict which viruses that could theoretically acquire genetic material from GM crop plants would have altered pathogenicity. (Although the same argument applies to naturally evolving strains, mutant and recombinant viruses).

The view that the transfer of virus-derived transgenic DNA or RNA to viruses is inevitable is based on laboratory experiments in which defective viruses were artificially inoculated onto GM plants that contained restorer viral transgene sequence which then regenerated viable (wild-type) virus (Lommel and Xiong 1991; Greene and Allison 1994; Greene and Allison 1996; Borja *et al.* 1999; Adair and Kearney 2000; Varrelmann *et al.* 2000; Schoelz and Wintermantel 1993; Wintermantel and Schoelz 1996; Gal *et al*. 1992; Frischmuth and Stanley 1998).

In fact all published examples of stable recombination between a defective (non-viable) virus and a homologous viral transgene contained in a GM plant have been achieved using experimentally designed, laboratory-based systems. In every successful case, a selection pressure was engineered into the test system in order to restore viability to wild-type or near-wild-type levels in any recovered virus through recombination rather than simple genetic reversion.

It is important to note that, although laboratory experiments demonstrate that viral transgene transcripts can be available for recombination, there is no evidence to suggest that such recombination events take place in the absence of suitable selection pressure. For example, a large-scale 6-year field trial with potato plants expressing the CP or replicase genes of potato leafroll virus (PLRV) provided no evidence for any modifications in transmission, transcapsidation, or synergism with any virus that could infect the potatoes (Thomas *et al*. 1998). Similarly, large-scale field introductions of coat-protein transgenic virus-resistant papayas in Hawaii have had no negative pathological or ecological impacts such as HGT creating new or devastating viruses (Ferreira *et al.* 2002).

There are no reports from laboratory or glasshouse trials, or in commercial use, of any infectious wild-type virus picking-up any transgene of viral (or any other) origin, or indeed picking-up any host RNA. On an evolutionary time-scale, a small proportion of a few atypical strains of plant viruses do appear to have incorporated small fragments (c. 70-119 nucleotides) of various cellular RNAs such as a chloroplast tRNA (Matsuta *et al*. 1992), or an exon from a chloroplast mRNA (ORF196; Mayo and Jolly, 1991), and possibly even a homologue of a nuclear heat-shock protein gene (hsp70) in a closterovirus (Karasev 2000). These examples appear to be rare and possibly unique. Modular sequence evolution in the generation of plant viruses became apparent in the 1980s and 1990s as complete sequence data accumulated.

Clearly, since the commercial purpose of a virus-derived transgene is to render the GM crop resistant (ideally immune) to infection by the target virus, any opportunity for homologous RNA-RNA or DNA-DNA recombination is greatly reduced (ideally to near zero). And, by definition, any unrelated virus to which the GM plant remains susceptible, will be unable to recombine through homologous template switching/copy choice mechanisms with an RNA copy of the original viral transgene. Non-homologous recombination is even less efficient.

There have been no published or anecdotal reports of recombination between viral transgene(s) and natural (wild-type) viruses during large-scale GM crop field trials (many

thousands since 1987), or in commercial-scale cultivation [e.g. all Hawaian papayas since 1997 (Gonsalves *et al*. 2002); US yellow crookneck squash (Asgrow/UpJohn Co.) since 1995; potato leafroll virus-resistant "New Leaf" potatoes (Thomas *et al.* 1998) since the early 1990s].

Any viral transgene that produces a functional or dysfunctional protein which failed to generate field-level resistance [for example, through spontaneous mutation (loss of function)], or somehow exacerbated the severity of an infection by an otherwise mild strain of the same virus (or an unrelated virus), poses an economic risk to the farmer, the seed producer and the biotechnology company - but not to the environment or to consumer health or safety. If they occurred, such events would render a particular GM crop variety commercially useless. They do not however result in any stable genetic change to the primary target virus or its progeny. As with transcapsidation, such events represent a genetic and epidemiological 'dead-end'. In fact, one genus of plant viruses, the Umbraviruses, relies on transcapsidation of their RNA-only genome in the coat protein of a co-infecting Luteovirus in order to move from plant-to-plant. In short, any loss of crop protection, or deleterious agronomic trait arising from a viral transgene, or its RNA or protein product acting in *trans* may pose an economic problem for commercialisation of that particular GM crop line, but it is not an HGT issue. As described later, under 'Likely future developments' and 'Technological and regulatory approaches" (and in Tepfer, 2002), detailed studies of the mode of action of viral (trans)genes has made it possible to eliminate proposed potential sources of potential risk such as transcapsidation or altered transmission by mutating the coat protein transgene.

However, most examples of viral transgene-mediated plant resistance operate through signalling rapid degradation of the incoming viral genome by a natural plant defence pathway (analogous to post-transcriptional gene silencing/cellular mRNA degradation). If a second virus infected a GM plant and encoded a suppressor of PTGS of the primary target virus from which the transgene was derived, then the efficacy of the original transgene may be compromised. Although not relevant to HGT, such an event could create an economic risk during commercialisation. Obviously, those who develop viral GM crops should test each transgenic line with viruses that may co-infect the plant in the field, especially those known to act synergistically with the primary target virus in nature (i.e. to suppress PTGS). Evidence for such an interaction was published by Barker *et al*. (2001).

The horizontal transfer of genetic material from a GM plant to a super-infecting virus can be achieved in the laboratory using model recombination-selection systems, but remains a hypothetical situation in the field. Given that the detail and origin of any viral transgene sequence will be known, predicted or even speculative risks can be avoided. Thorough screening, experimentation and analysis prior to any experimental or commercial field release would be required to test the efficacy, durability and stability of the new transgene and detect any predicted or unpredicted consequences.

Our understanding of the potential for virus-derived transgenes to recombine with and transfer to viruses is based on a substantial evidence base. The general questions asked by scientists working in this area and recent peer reviewed publications addressing them are described below:

Does RNA recombination between virus-infected transgenic plants transformed with portions of viral genomes occur?

Whether RNA recombination in virus-infected transgenic plants transformed with portions of viral genomes could potentially generate novel viruses with biological properties distinct from those of parent strains has been considered over 15 years. The strategy widely used to investigate this possibility is to apply a strong selection pressure to ensure that any virus generated as a result of a recombination event between a (defective) virus and a transgene has an advantage over the virus used for inoculation. The experimental procedure used by several authors has involved inoculation with a movement-deficient mutant (non-viable) virus (usually with a deleted or non-functional coat protein gene) onto transgenic plants (in most cases *Nicotiana benthamiana*) that expressed a gene encoding the corresponding part of the viral genome (including the coat protein gene) in a functional form. Systemic movement of symptoms then indicates recombination in that particular plant. Recent examples of this type of experiment are described in Box 7.3.

Box 7.3.

When *N. benthamiana* plants transformed with a non-translatable 3'-half of the tobacco mosaic virus (TMV)-GFP genome (from part of the RNA polymerase to the 3'-untranslated region) were inoculated with a TMV coat protein mutant, which could not move efficiently through the host, recombinant RNA was detected in 32% of inoculated plants. Nevertheless, the resulting recombinants were less fit than wild-type and no encapsidation of the recombinant viral RNA was detected (Adair and Kearney, 2000).

Wild-type plum pox virus (PPV) was restored in transgenic *N. benthamiana* plants that expressed the PPV coat protein with a complete 3'-non-translated region when inoculated with either a CP-deficient PPV, or a chimaeric PPV with CPs derived from other potyviruses (Varrelmann *et al.* 2000).

Recombination between an infecting virus and a transgene derived from a different viral species or strain cannot be ruled out. It was shown that the viral RNA-dependent RNA polymerase of several potyviruses and tomato aspermy virus have an ability to recognize heterologous 3'-untranslated regions (UTRs) included in transgene mRNAs (Teycheney *et al.* 2000).

The 3'-UTR adjacent to the capsid protein gene is frequently included in the construction of coat protein-mediated virus-resistant transgenic plants. Recombination frequencies between transgenic RNA and viral RNA can be reduced significantly by omitting or disrupting the 3'-UTR. This was shown using transgenic *N. benthamiana* plants transformed with the cowpea chlorotic mottle virus coat protein gene with or without its 3'-UTR (Greene and Allison 1994, 1996) which is the natural replication signal.

One report describes a rare double recombination event (i.e. not needing the 3'-UTR signal) leading to restoration of a wild-type viral RNA genome. Wild-type tomato bushy stunt virus (TBSV) was regenerated by a double recombination event in *N. benthamiana* plants transformed with the wild-type TBSV coat protein gene and infected with a mild-symptom TBSV mutant containing a defective coat protein gene. Similarly the TBSV-CP was restored when TBSV-CP transgenic plants were inoculated with a chimeric cucumber necrosis virus (CNV) containing the defective TBSV coat protein gene (Borja *et al.* 1999).

Does DNA recombination between virus-infected transgenic plants transformed with portions of viral genomes occur?

Similar to RNA recombination, there are examples of DNA recombination between a plant DNA virus and a viral transgene. Successful recovery of nearly wild-type geminivirus African cassava mosaic virus (ACMV) as a result of a recombination event between a CP-deletion mutant of ACMV and an ACMV CP transgene was reported in *N. benthamiana* (Frischmuth and Stanley 1998).

What effects could interactions between viruses and transgenes have?

It is theoretically possible that interaction between a virus and a transgenically expressed heterologous proteins could increase symptom severity as a result of synergism**,** as occurs in some natural pairwise mixed infections in non-GM plants. Cases where this happens, and the protein responsible have been elucidated experimentally over the past 15 years.

It is also possible that an otherwise non-transmissible virus could become packaged in the coat protein of another virus expressed at low levels in a transgenic plant (transcapsidation) and then be able to be moved by insects etc. to another plant. This will not result in heritable genetic change and is believed to be an epidemiological 'dead-end'.

It is possible that a transgene which signals 'silencing' of an incoming target viral RNA may cease to function if the plant is infected by a virus that can suppress (overcome) RNA-mediated resistance (i.e. suppress gene silencing). It was shown that when *N. benthamiana* plants were transformed with potato leaf roll virus (PLRV) full-length cDNA, only a minority of mesophyll cells accumulated virus. When these plants were then inoculated with potato virus Y or a tobacco mosaic virus-vector that expressed the potyviral PTGS suppressor protein P1-HCPro, the proportion of cells that showed PLRV replication increased dramatically (Barker *et al.* 2001).

In the debate on the safety of GM plants that express viral sequences it has been claimed, that the CaMV 35S promoter poses some alarming risks. It was proposed (Ho *et al.* 1999) that this promoter could recombine to activate dormant viruses, create new viruses, and cause cancer by the overexpression of normal genes. A full and measured response to these claims has been published (Hull *et al.* 1999) and there has been further discussion on the Review website (ISIS[38,39]; Roger Morton[40,41]). Ho *et al*. cite a paper by Kohli *et al.* (1999) in support of their theory – in this study several genes under the control of the CaMV 35S promoter were bombarded into rice and became integrated into the genome by DNA repair-mediated recombination. Although only a small number of transformed rice lines were studied, the authors showed that when integration occurred by recombination within the 35S promoter, one site was frequently involved. However, the 35S promoter itself was not any more likely to recombine than any other part of the DNA construct. Nevetheless, Ho *et al.* (1999) claimed that the 35S promoter would be promiscuous and mobile in the plant genome (like a transposon). They also proposed that such mobility would permit the CaMV promoter to insert into the genome of any organism that consumed the transgenic plant's DNA with adverse consequences. The scientific evidence does not support this reasoning:

[38] http://www.gmsciencedebate.org.uk/topics/forum/0030.htm
[39] http://www.gmsciencedebate.org.uk/topics/forum/0067.htm
[40] http://www.gmsciencedebate.org.uk/topics/forum/0062.htm
[41] http://www.gmsciencedebate.org.uk/topics/forum/0079.htm

(a) there is no evidence that the CaMV 35S promoter is mobile, unlike natural and widespread plant transposable elements. Quist and Chapela (2001) reported the fragmentation of the CaMV 35S promoter in maize landraces when transgenes were transferred from GM maize. However, the design of the experiments on which this particular conclusion was drawn was deeply flawed (Kaplinsky *et al*., 2002; Metz M. and Fütterer, 2002).

(b) we eat large amounts of CaMV-infected crucifers (a 1980's study showed 10% of UK cauliflowers and cabbages were infected. Organically grown crops are likely to have even higher levels). Each plant cell typically produces 100,000 virus particles, and hence 100,000 copies of the 35S (and 19S) CaMV promoter. Thus throughout evolution, humans have consumed plant viruses, or have eaten animals which have themselves consumed plant viruses. There is no evidence for integration or recombination with our genomic DNA. Evidence supporting the view that CaMV DNA does not pose any novel cancer risk is discussed further in a contribution to the website (Professor D. Murphy[42]). Please also refer to Chapter 5.4: *the fate of transgenic DNA in GM plants*.

(c) in addition to CaMV-infected vegetables and transgenic crops containing CaMV 35S, all banana varieties that have been studied contain multiple copies of another Pararetrovirus - banana streak badnavirus – naturally integrated into their genomes. Despite exposure of humans to these Pararetrovirus DNA sequences and promoters there is no evidence for any ill-effects from newly emerging viruses or cancer genes, even in Uganda where bananas are the staple diet and HIV (a Retrovirus) is rife.

7.5.4 Is there general scientific agreement?

There was consensus among scientists at a USDA Workshop 'Assessing the Risk of Plant Viral Transgenes' (1995). It was concluded that there was no rational or conceptual reason to assume that any particular plant viral insert has an increased potential for viral RNA recombination events. Expression of any fully functional viral protein such as a coat protein, cell-to-cell movement protein, replicase enzyme, suppressor of gene silencing or protein involved in the transmission of a particular plant viruses by its associated insect, fungal, mite or nematode vector obviously has the potential to act *in trans* to complement a defective strain of the primary virus, or even a secondary virus – as can happen in any natural mixed field infection (e.g. synergy). Such phenomena do not, however, lead to HGT.

7.5.5 Is the issue unique to GM?

With the exception of the Badnaviruses mentioned above, plant viral sequences do not occur naturally in plant genomes. Thus, even the hypothetical risk of recombination and gene flow from a natural viral insert in a plant genome to another (un)related invading virus is zero. However, gene transfer, recombination, genetic reassortments, complementation, synergy and transcapsidation can and do happen in natural mixed virus infections in a wide range of plants. Moreover, opportunities for such events have been increased over the last 30 years, since the phenomenon of cross-protection (first reported in 1929 by McKinney) has been

[42] http://www.gmsciencedebate.org.uk/topics/forum/pdf/0011.pdf

widely deployed in several major glasshouse and field crops. Here, a natural or artificially generated mutant mild strain of an otherwise virulent natural virus is applied to the crop to 'protect' each plant against subsequent natural infection and hence devastating symptoms. Occasionally, the mild (protecting) virus strain mutates or reverts spontaneously to a more devastating form, or recombines with another infecting virus with serious consequences. For example, Brazilian orange trees normally 'cross-protected' using a mild strain of citrus tristeza virus have recently become decimated by a new CTV strain. Similar risks are present in glasshouse tomato and cucurbit crops that are "cross-protected".

7.5.6 Are there gaps in our knowledge or scientific uncertainties and are these important?

No good data exist on how the strength (however this might be measured) of a particular genetic/fitness selection pressure could affect viral (or other) genome recombination rates. However while (difficult) experiments to study this may provide useful genetic and evolutionary data, better understanding and some reference points, they may be of academic interest compared with practical experiences and experimental data already accumulated during extensive GM crop trials and commercial plantings. For example, Monsanto's 'New Leaf' potato was the subject of an extensive 6-year study (Thomas *et al*. 1998). Over 25,000 plants in 442 lines transformed with 16 different coat protein gene constructs (with a Luteovirus replication origin), and 40,000 plants in 512 lines transformed with 7 different replicase gene constructs of potato leafroll virus were exposed to field infection over a 6-year period. Individual plants were inspected annually, and extensive molecular and biological studies done on any PLRV or heterologous viruses found to be infecting the crop. No changes in virus properties or evidence of recombination (HGT) were found.

The probability of occurrence of any plausible hazard occurring through homologous or heterologous recombination between a virus and viral transgene-derived genetic material may be influenced by the scale of any commercial release. However, as in nature and laboratory experiments, almost all recombinant viruses are unlikely to survive or to dominate the population against competition from the wild-type parent viruses.

As in the later stages of conventional breeding and selection of a new non-GM crop plant variety, the inheritance, stability and functional utility of any new trait (whether GM-derived or not) should be assessed under all probable environmental conditions and agronomic practices. Any loss of function poses economic and efficacy problems that would render the new variety non-profitable, but does not raise HGT issues.

7.5.7 Likely future developments

Recent progress in increasing our understanding of the mechanism of induction of PTGS has led to the development of new and more targeted strategies to induce RNA-mediated resistance to viruses (Vaucheret *et al.* 2001; Chicas and Macino 2001; Hannon 2002). Specific constructs designed to express double-stranded (ds)RNA corresponding to parts of the target virus genome has proved very efficient in inducing protection against the whole virus. For example, barley plants transformed with a single copy of a construct designed to produce a hairpin RNA from barley yellow dwarf virus-PAV (BYDV-PAV) showed strong

heritable resistance to BYDV-PAV. This protection was rated as immunity. The virus could not be detected by ELISA methods, even in those plant tissues challenged with virus inoculum, or be recovered by aphid feeding experiments (Wang *et al*. 2000).

It has been known for some time (Voinnet *et al.* 1999) that some virus-coded proteins can suppress PTGS and thereby overcome a plant's defence mechanism. When this happens *in trans*, between two viruses in one plant, it is called 'synergy'. The impact of natural viral synergy-type interactions operating against target viral transgene-mediated resistance in a field crop are largely predictable, yet the phenomenon has not yet been reported (Tepfer 2002). Nevertheless, although not directly relevant to heritable HGT, the area probably merits further careful analysis both for commercial reasons as well as to increase our knowledge. Clearly, care is required to avoid stacking in one plant of viral gene sequences with the ability to complement or act synergistically with one other or with common field viruses in the locality.

Another opportunity to design virus-resistant transgenic plants involves expression of mutant forms of viral proteins, which interfere with viral infection. For example, transgenic expression of a tobacco mosaic virus (TMV) coat protein mutant (CPT42W) resulted in very high levels of resistance to TMV. This was due to interference by the mutant CP with the normal movement protein production and subsequently with cell-to-cell movement of the virus (Bendahmane *et al*. 2002)

Transgenic resistance to viruses may also be induced by transforming plants with non-viral genes. Broad resistance to a variety of plant RNA viruses was reported in tobacco plants transformed with the gene for human dsRNA-dependent protein kinase (PKR), placed under the control of a plant wound-specific promoter. In human cells, PKR confers resistance to viruses by inhibiting their replication by inactivating the translation initiation factor, eIF-2α, following activation by dsRNA. Transgenic plants expressing the PKR gene showed significantly reduced viral symptoms, or no viral symptoms at all, when challenged by different plant RNA viruses, such as cucumber mosaic virus, tobacco etch virus, or potato virus Y (Lim *et al.* 2002).

Weeds frequently act as over-wintering reservoirs for viruses that can then re-infect a genetically unrelated crop the following year, provided they share some suitable vector to transfer the virus. *A priori*, there is no reason that the seasonal crop host should be sexually compatible with the virus-susceptible over-wintering weed species. Nor that weeds that may be able to acquire a viral transgene by cross-pollination with a GM crop are necessarily hosts for the same virus, or even that such an event would provide any evolutionary advantage (e.g. increased weediness). With the exception of very few pollen transmitted viruses, plant-to-plant gene flow and virus transmission are two completely unrelated and independent events. Indeed, if a weed was both a host for the target crop virus and sexually compatible with the crop, then hybridisation and transfer of the resistance trait would render the weed resistant to the virus. This would then reduce the viral inoculum pressure next year by lessening the viral reservoir. As described previously, crossing with the weaker crop genome would also reduce the overall competitiveness or persistence of the weed (see section 7. 3 where linkage drag is discussed in more detail).

7.5.8 Where there is important scientific uncertainty what is the potential way forward?

Research

If some suitably precise and sensitive experimental system could be devised, then it may be instructive to compare the frequency of recombination events in natural mixed virus infections with events in a single virus-infected GM plant.
If, or when, a viral transgene mediated resistance trait breaks down, or high field inoculum pressure overcomes GM protection, are spontaneous mutants in the ("quasi-species") population being preferentially selected and do they persist?

There is always a drive to further refine the parameters to design more effective RNAi transgenes to target PTGS at the incoming viral genome(s). It would also be valuable to achieve broader spectrum resistance against related viruses.

Just as different viruses could reduce their propensity to recombine or reassort genome segments with co-infecting viruses during mixed infections, we know relatively little about the relative compartmentalisation of transgene transcripts and their target viral RNAs. PTGS and dysfunctional protein-mediated strategies that generate functional field resistance in GM crop plants may therefore not provide effective opportunities for efficient template switching and recombination/HGT.

Technological and Regulatory Approaches

Several practical recommendations can be made to minimise any even the theoretical risk of adverse effects. In an earlier report (DETR 1999), key safety features in designing transgene constructs included:

(i) minimising the length of any virus-identical, homologous sequence and avoiding origins of RNA or DNA virus replication such as genomic or sub-genomic RNA promoters
(ii) including multiple dispersed point mutations (i.e. non-revertible and translationally silent if necessary) in any potential protein-coding sequences to render them dysfunctional or any possible recombinant genome defective
(iii) omitting insect/fungus/nematode transmission signal sequences on coat proteins
(iv) focussing on RNAi strategies and
(v) avoiding hyper-mutable molecules such as defective interfering (DI) or satellite RNAs.

For example, to eliminate any possible risks related to vector transmission of non-transmissible viruses as a result of transcapsidation, point mutations can be introduced into the coding sequence of the coat protein transgene to eliminate its ability to package RNA. Thus *N. benthamiana* plants were transformed with a plum pox virus coat protein gene with a deletion of the amino acid triplet (DAG) involved in aphid-transmission. Experiments demonstrated that the modified form of the PPV coat protein in transgenic plants provided good control, without any potential biological risk associated with transcapsidation and spread (Jacquet *et al*. 1998).

Knowledge-based agriculture, evidence-based regulations and open-minded decision-making depend on facts and weighing-up benefits and risks. GM crops that express viral sequences exploit a natural plant defence pathway to target many of the otherwise intractible viral pathogens. Viruses greatly reduce crop yields, blemish products and require farmers and growers to rely on pesticide sprays, chemical fumigants or steam/flame soil sterilisation methods to remove their insect, fungal or nematode worm vectors. We should judge GM crops against these options.