

Gene Flow from GM Plants

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Gene Flow from GM Plants

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Preface

The development of GM crops has stimulated intense research interest in the possibility of gene flow between these plants and their wild or weedy relatives. Unfortunately, in spite of the investment of considerable effort and money in these activities, we are still too often unable to quantify the risks of ecological damage associated with gene flow. This is due partly to the huge breadth of knowledge required to assemble a comprehensive risk assessment. For instance, many scientists active in research on the mechanics of gene flow nevertheless lack a deep understanding of what is required to identify, characterise and assess ecological risk. Conversely, many of those who are aware of the risk assessment process and the framework used for legislation have insufficient knowledge of the reproductive biology, agricultural systems, modelling and ecological literature required to compile a balanced assessment of risk. There is a need therefore for a holistic source of reference that brings together the knowledge and information required for the risk assessment of gene flow from GM plants, and allows us to explore the possibility of managing risk. This book combines the expertise of all the various stakeholders, allowing readers to view the whole jigsaw. It will also serve as a manual for assessment, measurement and management of the various categories of risk associated with gene flow from GM plants.

The book is structured in three sections. Section 1 (Chapters 1 and 2) sets the scene, section 2 (Chapters 3–8) focuses on identification and quantification of risk, and section 3 (Chapters 9 and 10) focuses on risk management. It is important to see where science fits into the “GM debate”, and the initial chapter describes the UK case study of the GM Nation debate and the GM science review. The diversity of GM crops/traits is often ignored in the black and white discussions about the merits and potential pitfalls of biotechnology. The power of recombinant DNA technology is evolving rapidly and the new developments (Chapter 2) may affect the cost/benefit analysis and regulatory process (see Chapters 9 and 10). Identification and quantification of the risks associated with gene flow require knowledge of the mechanisms of pollen dispersal (Chapter 3) and hybridisation events (Chapter 4). Details of how to measure rare hybrid events (Chapter 5) and the ecological fitness costs (Chapter 6) are equally essential if one is to be more quantitative in risk assessment. Since risk is a function of hazard and exposure, prioritization of the hazards associated with gene flow (Chapter 7) and quantification of exposure levels to the “hazard” (Chapter 8) are essential components of any risk assessment framework. Management of the risks associated with gene flow requires stringent regulation (Chapter 9), a process which is frequently misunderstood by non-regulators. However, if the number of GM crops requiring regulation increases, there is a need to adjust the ways in which both the

risk assessment is conducted and the data generated are used to guide regulation (Chapter 10).

We currently stand at a threshold. Gene flow from GM crops poses risks at many levels, but the use of GM crops can also bring many benefits. The cost/benefit analysis requires significant information, which in many cases is not available. In this book, we have tried to provide comprehensive coverage of the scientific, regulatory and management issues relating to gene flow from GM crops. Undoubtedly there is a pressing need to increase crop productivity, especially in parts of the world where pests, diseases and other stresses are significant. The use of GM crops has the potential to be a vital tool in the toolbox available to address these problems, but issues such as gene flow mean that their use will never be risk-free. This book has been written to inform readers and to allow them to make their own judgments on how best to proceed.

We would like to take this opportunity to thank the authors for their work in producing chapters of such a high standard – and in some cases on time! We would also like to thank Graeme MacKintosh and David McDade of Blackwell Publishing for their support and “gentle pressure” throughout the development of this book.

Guy Poppy and Michael Wilkinson

1 Where science fits into the GM debate

Philip J. Dale

1.1 Background

The science of plant breeding has advanced significantly over the past 80 years, and during this time there have been many important innovations (GM Science Review, 2003). GM methods of plant breeding, developed over the past 20 years, allow us to isolate genes from different classes of organisms (unrelated plants, microbes, animals) and incorporate them into a wide range of crop plants. The land area covered by GM crop cultivation increased steadily from the first small-scale field experiments in 1986 to over 67 million hectares grown worldwide in 2003 (James, 2003). About 20 years ago, it was decided to introduce an additional tier of risk assessment for GM crops when compared with non-GM crops. Following this decision, there was a gradual evolution of regulatory oversight worldwide.

1.2 Regulation

The regulatory framework adopted for the assessment of GM crops varies in different countries, depending on whether they adopted new laws or adapted existing ones (Tzotzos, 1995). There are also differences in emphasis in deciding the triggers that bring regulation into play. In Europe, all plants and organisms modified by the direct uptake of DNA are regulated irrespective of the organism from which that DNA is obtained. In North America, greater emphasis is placed on the nature of the plant breeding product. However, the differences in practice are currently largely academic. All regulations recognise the importance of rational scientific analysis of each GM organism, case by case (GM Science Review, 2003, 2004). They recognise that from a scientific perspective, few, if any, generic judgements can be made about the safety and impact of GM crops compared with non-GM crops.

Even though there are differences in regulatory framework, the questions asked in risk assessment internationally are similar and include the following:

- What is the nature and function of the gene of interest in the donor organism?
- What is the effect of the introduced gene on the modified organism?
- Is there evidence of any change in toxicity or allergenicity in the modified crop?
- Is there evidence of a change in persistence or invasiveness in the modified crop plants?
- Are there impacts on (friendly) non-target organisms in the environment?
- What is the frequency and consequence of gene flow from the GM crop to sexually compatible weeds, feral plants and adjacent crops?

While adoption of the process of GM as the primary trigger for requiring a higher tier of safety assessment has merits, it also has weaknesses. The principal one is that very similar modifications (e.g. herbicide tolerance) achieved by GM and non-GM plant breeding, which raises comparable gene flow issues, are regulated very differently.

1.3 Stimulus for research

A significant consequence of the extensive risk assessment carried out on GM crops over the past 20 years has been the stimulation of research. The research community has been required to seek answers to questions about GM crops that have rarely been considered hitherto for any crop, irrespective of whatever breeding method was used to produce them.

Studies of gene flow and its consequences have formed an important part of the international research carried out to underpin the assessment of GM crops (see, for example, BBSRC, 2004; Kessler & Economides, 2001). Other relevant topics have included the following:

- The nature and characteristics of DNA insertion into plant genomes
- The stability and expression of introduced genes
- Gene promoter and terminator function and tissue specificity
- The impact of crops on agronomy and wildlife

As a result of about 15 years of GM-related research on gene flow, we now have a more comprehensive understanding of sexual compatibility between crops and related species, the degree of geographic association between crops and related species, the dynamics of pollen viability and dissemination, the success of hybridisation between plants at different distances and the opportunities for gene introgression over sexual generations between crops and other species in nature (see, for example, Lutman, 1999).

The high profile Farm Scale Evaluations (FSEs) involved assessment of the impact on wildlife of herbicide treatments associated with the cultivation of particular GM herbicide tolerant crops (Royal Society, 2003). A minor part of that programme studied gene flow by pollination, but the primary objective of the FSEs was to compare the environmental impact on wildlife of agronomic management practices associated with each GM crop and a comparable non-GM crop of the same species. These experiments raised important questions about the appropriate balance between the provision of weeds to feed wildlife and the production of agricultural crops. The FSE experiments were initiated to address specific questions about the impact of GM crops on wildlife, but ultimately raised fundamental questions about how we manage the competing aspirations for the use of farmland and the wider environment. I shall return to this topic in the discussion section.

This extensive biosafety research, stimulated by a desire to base a regulatory process on sound science, has been interpreted in different ways by those with an interest in the future commercialisation of GM crops. On the one hand, many within the regulatory processes view the extra research and regulation as a responsible expression of the precautionary approach applied to modern plant breeding. On the other hand, some in the public debate argued that if it is considered necessary to carry out a great deal of expensive additional research to assess the safety and impact of GM crops, they must be fundamentally different and innately more uncertain than non-GM crops. A classic catch-22.

1.4 Vigorous campaigning

It is fair to say that before and during the national public debate in the United Kingdom, there was vigorous campaigning on the commercial future of GM crops. It is also reasonable to state that the majority of campaigning over several years has been against the cultivation of GM crops by activist groups and by certain sections of the press. Some of this activism included vandalism of scientific field experiments designed to provide important data on the impact of GM crops compared to their non-GM counterparts (Elliott, 2003). The campaigning has been to an unprecedented degree for any recent scientific advance associated with agriculture. Throughout the history of plant breeding, there have been many developments, including mutagenised crops, polyploid crops, the use of wide hybridisation between normally sexually incompatible species and a number of other significant developments. Several of these developments carried very high levels of unpredictability that had to be addressed during testing and evaluation of the new crop varieties produced. But opposition to these methods was not adopted by campaigning organisations at the time, and the developments received little or no public or press attention.

The vigorous and polarised campaigning associated with GM crops frequently led to expressing the issues, including the science, in a manner intended to stimulate the greatest political, judicial and press impact (Dale, 2004). Regular use in the media of the terms 'Frankenstein food', 'mutant crops' and 'genetic pollution' is evidence of this, and has branded GM crops in the minds of many people as innately undesirable and even dangerous.

Negative campaigning has of course been common in the history of many areas of innovation and unfamiliarity, from smallpox vaccination to stem cell research, from steam engines to mobile phones. The early canal pioneers in the United Kingdom over two centuries ago were branded as 'carving up the countryside with stinking sewers'. While precaution in research and development is essential, so is an appropriate opportunity for innovation and advancement.

This, therefore, was the backcloth to the public debate on the commercial future of GM crops held in the United Kingdom during 2003. It was the context within which science was expected to inform and underpin the debate.

1.5 The GM Nation Public Debate

The public debate was managed by a steering board with members holding a diverse range of opinions on GM crops. Members ranged from a company developing GM crops commercially to the manager of a campaign organisation committed to preventing the commercialisation of GM crops. This diversity of view within the steering board made its operations particularly challenging. The aims of the debate were:

- To identify and focus on grass-roots opinion within the general public
- To avoid polarisation as far as possible
- To develop a wholly open and transparent process
- To provide a process that is evidence based
- To provide an opportunity for members of the public to debate openly and to reach their own informed judgement
- To allow the questions raised by the general public to shape the course of the debate

In addition to overseeing the organisation of the public discussions, the steering board had to agree on the information to be given to the general public about the science and future potential use of GM crops. As one of the few scientists on the steering board, it was particularly difficult to accept that scientific information, accumulated over decades of research and verification, was categorised by some as a scientist's view borne out of vested interest. Evidence, for example, that the vast majority of the scientific community would consider self-evident (e.g. the central role of DNA in inheritance) was by some given the same value as an idea based on little or no evidence (e.g. a link between GM crops and severe acute respiratory syndrome, or SARS).

Issues relevant to the widespread cultivation of crops can be complex and difficult to communicate in public discussions by sound bites. Another difficulty is that people have become disconnected from agriculture and the origins of their food (Curry Report, 2002). As a result, standard non-GM practices in plant breeding and agriculture often came as a surprise in public discussions. This emphasised the importance of greater dialogue between scientists and members of the public, but made it difficult in the debate to place GM crops in an appropriate agricultural and plant breeding context.

The fact that the debates were carried out in the midst of intense anti-GM campaigning by activist groups and sections of the press also made it particularly difficult to have an informed, balanced and dispassionate discussion. While there were significant pockets of activity and involvement in the debate from the scientific community, many scientists appeared to find polarised argument and sound-bite communication uninviting and even futile. Well-informed and balanced dialogue was often the casualty.

1.6 Gene flow issues raised in the public debate

The frequency of pollination between crops and adjacent sexually compatible species at different distances is an important issue in the scientific assessment of the impact of GM crops, but precise frequencies of pollination were rarely discussed in the debates I attended. The concept of gene flow was rarely expressed in these terms, but rather as concerns about contamination and environmental damage. The salient issues raised are discussed as follows.

1.6.1 *GM is unnatural*

A common view of those opposing commercialisation of GM crops was that GM plants are unnatural and alien and therefore any level of gene flow is unacceptable. There was the feeling among some participants that to move genes into crops from unrelated plants, microbes and potentially from animals was fundamentally unacceptable. Discussions about the remarkable similarity of genes from very different organisms and kingdoms, and questions of what defines a wheat gene, a bacterial gene or a viral gene, were again difficult to engage within the often highly charged atmosphere.

Comparison with crops bred by induced mutagenesis was raised by scientists contributing to the debate, to illustrate the successful and safe use of a plant breeding method that frequently has a higher level of unpredictability than GM. In practice this was a comparison difficult to communicate to those unfamiliar with the practicalities of genetics and plant breeding.

1.6.2 *Genetic contamination*

The issue of cross-pollination with GM crops was most intense in relation to compatibility of GM crops with organic farming. The organic sector has decided that there is no place for GM crops in organic agriculture. While many agriculturalists believe there are compelling scientific and environmental arguments in favour of the use of pest- and disease-resistant crops to reduce or eliminate the use of chemical sprays in agriculture, including those used in organic agriculture (e.g. copper-based fungicide sprays), the organic sector has decided for the foreseeable future to prohibit GM crops from organic agriculture. There are different ways of interpreting this prohibition: on the one hand, as not allowing the deliberate cultivation of GM crops; and on the other hand, as zero tolerance of the presence of any GM plant material in the vicinity of an organic farm.

Interestingly, the certifying bodies for organic agriculture accept a pragmatic view when considering coexistence with certain kinds of farming. There is, for example, tolerance of a level of spray drift from neighbouring farmers. There is derogation in the feeding of organically produced animals when organically produced feed is in short supply (e.g. during the UK foot and mouth disease outbreak).

There is also a general tolerance of the movement of noxious weed seeds, pests and diseases between different farming systems. Some campaigners for organic agriculture, however, have argued for zero tolerance of the presence of any GM in organic crops. If successful, this would make it difficult for organic and GM agriculture to coexist. To my knowledge, however, no organic farmer in the United Kingdom to date has lost his or her organic price premium following production of an organic crop in the vicinity of a GM crop.

The fundamental difficulty is that one farming system, in this case the organic sector that occupies about 4% of the UK agricultural land area (including land in conversion, but excluding common grazing; AEBC, 2003), is attempting to define its product in a way that will prevent, or at least severely inhibit, the viability of any farmer wishing to grow a GM crop. The principal campaigners for organic agriculture argue that the consumers of organic food demand the absence of GM content. This is not independent of vigorous campaigning against GM crops and foods on their behalf, and the threat of removing the organic premium from farmers who produce crops containing GM material. Defining organic produce in this way is not without significant commercial interest on behalf of the organic sector.

1.6.3 GM and organic agriculture cannot coexist

Although some opponents of GM crops argue for zero tolerance of the presence of any GM plant material in a non-GM crop, in practice there are no analytical methods capable of proving zero GM content. The limits of routine detection of the presence of GM material are generally considered to be around 0.1%, that is, one GM seed in 1000 non-GM seeds (AEBC, 2003). While this level of analytical resolution is feasible in a laboratory context, in agricultural practice, it will be difficult to stay below this threshold when GM crops are grown in widespread agriculture. This is because the adventitious presence of GM crop material can result from several sources in practical agriculture, including:

- The seed sample used to establish the crop (seeds are often multiplied abroad in countries where GM crops are commonly cultivated)
- The GM seeds remaining in seed drills, combined harvesters, transportation machinery and grain silos
- The GM seeds and plants found as volunteers in the field from previous cropping or are carried there by animals and machinery
- The GM seeds resulting from gene flow by pollination from a GM crop

While the 0.1% threshold is currently the self-imposed aspiration of certain sectors of the organic movement, the only level that has statutory authority in the European Union (EU) is the 0.9% threshold used for labelling purposes. Any crop that contains more than 0.9% GM content must be labelled as containing GM material. A crop with less than the 0.9% threshold need not.

1.6.4 GM crops will damage the environment

Another issue raised in debates was the potential consequences of the transfer of genes introduced by GM breeding to natural habitats and wildlife. The concept of environmental impact from gene transfer was often referred to in generic terms as being inherently damaging to the environment. Concerns were frequently mentioned about the production of superweeds as a result of the transfer of herbicide tolerance genes from GM crops to weeds.

1.7 Findings of the debate

The main conclusions from the debate can be found elsewhere (GM Nation, 2003), as can be a detailed account of the lessons from the debate process (Understanding Risk Team, 2004; Defra, 2004). Some quotations from the report give important insights into the perspective given to scientific information in the debate process.

- ‘It was not part of our intentions (the Steering Board) in this report to say whether the public were right or wrong about any GM issue, even on matters of fact’ (Introduction to the Executive Summary).
- ‘It (GM) is not only an issue in its own right but acts as a proxy for many other current concerns which provoke strong feelings’ (Paragraph 45).
- ‘There was a broad desire to know more and for further research’. ‘They (some participants in the debate) want a corpus of agreed “facts”, accepted by all organisations and interests’ (Paragraph 5).

Often discussion began with GM crops but rapidly moved to broader issues around agriculture, the environment and governance. Although there was a general desire through research to know more about GM crops, the discussions, especially in large meetings, were rarely conducive to a detailed consideration of the current state of scientific knowledge. There was also little opportunity to discuss specific research needs for the future.

1.8 Discussion

In the heat of public debate, and its associated campaigning, it was easy to forget the context in which GM crops were being considered. Gene flow is a natural biological phenomenon that has been occurring between sexually compatible species since the beginning of agriculture. For some participants, they had already made up their minds and the science was almost irrelevant; they viewed the issue of the commercialisation of GM crops as principally ideological, ethical and/or political. In my view, to argue that GM crops are innately more hazardous than non-GM crops is like saying that a toxin gene from a bacterium introduced by GM is more hazardous than a deadly nightshade-type toxin introduced into cultivated potato by

pollination. Both are equally unacceptable and regulatory measures must be in place to prevent the use of crop varieties of this kind.

There were participants who were genuinely searching for a balanced and informed perspective, but this was difficult in the large structured meetings where there was often a preponderance of contributors from campaigning organisations. Interestingly, one participant at a large meeting said to his fellow participant, 'I came to this meeting to learn from well-informed people, rather than to discuss the issues with people who know little more than I do'. This has important lessons for future debates of this kind.

1.8.1 GM crops have become 'a lightning rod' for a range of concerns

The GM Nation report clearly acknowledges that GM crops have become a proxy for a range of concerns. The issues raised in discussion included impact on wildlife, the industrialisation of agriculture, the extensive use of chemical inputs, the perceived increase in power of multinational companies the globalisation of trade, the use of fuel to transport food across the world, the commercial future of organic farming and trust in the government. The GM public debate in the United Kingdom followed a period of vocal public opposition to the UK government for its involvement in the invasion of Iraq. The commercialisation of GM crops, therefore, seems to have taken on a significance in the activists' agenda and in the public's mind far beyond that relevant to a scientific consideration of the risks involved from the commercialisation of GM crops in the United Kingdom – crops that worldwide already cover over twice the land area of the United Kingdom.

1.8.2 Difficulty of holding a rational discussion of GM crops in context

In the broader public debate there was a marked lack of serious discussion about the broader agricultural context within which GM crops were being evaluated. The relevant broader issues include the following.

1.8.2.1 Method not mission

The debate concentrated on plant breeding method rather than on mission. A fundamental weakness of current reasoning on GM crops (and supported by the current EU regulatory process) is that similar plant breeding products with comparable gene flow and environmental impacts are viewed and regulated differently. For example, if a plant breeder produces a ryegrass variety that is tolerant to the herbicide glyphosate by both GM breeding and non-GM breeding, their regulation would be fundamentally different. The GM variety would require compliance with stringent assessment and regulation, probably costing hundreds of thousands of pounds. The non-GM variety, with similar potential environmental impacts, including those associated with gene flow, would require negligible comparable assessment or regulatory oversight.

An excessive preoccupation with the GM method also ignores the fact that there are significant advances being made in the efficiency of non-GM breeding that have the potential to produce novel crops. There is, for example, important progress in the targeted selection of induced mutations (Targeting Induced Local Lesions in Genomes – TILLING; Chapter 4 of GM Science Review, 2003), which, for some applications, may prove as powerful as GM plant breeding. This again highlights the question of whether assessment of the impact of gene flow should be determined by the breeding process or the nature of the breeding product. In my view, from a scientific perspective, a proportionate analysis of the characteristics of the breeding product should be paramount, irrespective of the breeding method used.

1.8.2.2 The FSEs raised wider issues

The Farm Scale Evaluations (FSEs) were a significant topic of discussion in the GM debate and the science review. While these were admirable, pioneering experiments and of great credit to the scientists involved, they present some significant challenges.

The experiments, as indicated earlier, evaluated the impact on wildlife of the herbicide treatment associated with cultivation of three GM crops, each modified to be tolerant to one herbicide. The GM crops were grown adjacent to a non-GM variety of the same crop, essentially using the variety and agronomic management the farmer would normally use, for comparison. The use of a comparable non-GM crop as a control is perfectly justifiable as a measure of statistical significance of the kind important for publication. The real difficulty is in establishing whether statistically significant differences are biologically significant in wider agricultural practice, where there is considerable variation in choice of crop and agronomic management. The main potential pitfall is that this type of ‘narrow sense’ control may be adopted by the EU regulatory process as a gold standard for assessing not only statistical significance but, more importantly, biological significance.

My concern is that a control of this kind provides no holistic yardstick that facilitates judgements about whether particular GM crops have an acceptable biological impact or not. If we are to use the narrow sense control, as in the FSEs, to judge the acceptability or otherwise of particular GM crops, then it would be logical to question all conventional crops and agronomic practices that have a greater adverse impact on the environment compared with these same FSE control crops. If we do not do this, then we are judging GM crops asymmetrically and unfairly against a highly subjective measure of biological impact.

As an illustration, the current regulatory position in the United Kingdom would support the continuous cultivation of winter wheat (non-GM) and judge it to be environmentally acceptable, but would prohibit the inclusion in the rotation of a GM oilseed rape break crop and would condemn it as environmentally unacceptable. In reality, the cultivation of continuous winter wheat is widely acknowledged as a major contributor to the reduction of birdlife over the past 30 years, whereas the inclusion of a break crop of GM oilseed rape would be relatively beneficial to wildlife. It is the whole farming system that has profound impacts on wildlife

and the environment, much more than single crops or treatments within it. If as scientists we do not question the use of these narrow sense measures of biological impact, then by default we legitimise them. Just as we are now critical of the decisions of former generations who removed trees, hedges and ditches and drove agricultural production at cost to the wider environment, so future generations will judge us for our narrow and blinkered perspective in assessing the current environmental impacts of particular GM crops. The challenge is to find measures of biological impact that operate at the level of the farming system, and make judgement accordingly.

1.8.3 Broader agricultural issues

It was also surprising that there was little mention within the GM debate of wider issues in agriculture. In the heat of the discussions on GM in the United Kingdom, the EU independently decided to reduce the area of set-aside land from 15% to 10%. This single change, arguably, has the potential to make a much more profound impact on the agricultural environment than commercialisation of the GM crops being debated. Another potentially significant event in the EU is the reform of the Common Agricultural Policy. This may provide an important opportunity to give incentives to farming systems that benefit wildlife and the wider environment, and practices that would benefit from future advances in the breeding of pest- and disease-resistant varieties.

1.8.4 Political context

There are many issues in which science and politics are uneasy bedfellows. It is clear that the debate about the commercial future of GM crops has become one of them. Engaging the public in a well-informed scientific dialogue, especially against a background of intense campaigning, is exceedingly difficult. It is important that society has access to the best science available, and as scientists we must be ready to discuss gaps in knowledge and areas of ignorance. We must also be more ready to discuss how precaution is used to manage risk. There is little science can do to resolve ideological differences except by providing underpinning knowledge to guide reasoning and discussion.

It is important that decision making about scientific innovation has a political element. Ultimately, politicians have to respond to their electorate. What is dangerous is when politics begins to influence the interpretation of science. Lysenko set back the science of genetics in Russia by several decades because idealism was used to interfere with the interpretation of science. Striking an intelligent balance between scientific logic and long-term political ideals is likely to present a challenge for a while longer until GM crop products have a clear and definable benefit to people, many of whom have little knowledge or empathy for the practical needs of agriculture.

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2 Crop biotechnology – the state of play

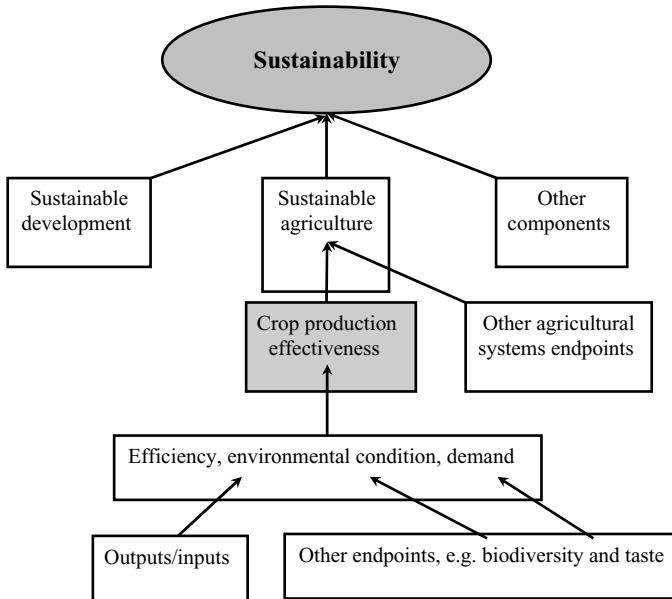
Thomas E. Nickson

2.1 Introduction

Among anthropogenic activities, agriculture is almost certainly the most important in terms of providing food, feed and fibre worldwide. Many types of agriculture are practiced today, including forestry, herding or livestock farming, aquaculture and crop production. Each kind is classified as either subsistence or industrialized, depending on the type and quantity of inputs and other factors (Raven *et al.*, 1998). Crop production is one form of agriculture that employs different practices, depending on whether an industrial or subsistence farming system is being used. Today's cropping systems are the outcome of thousands of years of coevolution within cultures, economic systems and environmental constraints. The 6+ billion people of the world rely on crop production for the vast majority of plant-derived necessities.

Agriculture, as it has evolved over the centuries and is practiced today, plays a major role in the current condition of the environment. In the latter half of the twentieth century, agriculture and agricultural practices came under intense scrutiny because of a growing environmental ethic, combined with an increased awareness of the linkage between food, feed and fibre production and the state of the broader environment. The widespread characterization of the anthropocentric view of the environment has been shifting from one of domination towards one of dependence and stewardship. Reasons for this change include a greater awareness that the environment provides the primary resources for successful agriculture, and that farming requires significant amounts of environmental resources, i.e. biodiversity, land, water and energy, which are in limited supply. In addition, the growing global population and shifting societal demographics have created a situation where fewer farmers are producing food for a growing number of consumers. Technological innovations have so far helped farmers to meet demand with improved environmental stewardship.

Looking to the future, two important questions facing society are: How will farmers produce sufficient food, feeds and fibre for the growing number of consumers, without jeopardizing the needs of future generations? And, recognizing that the needs of future generations require improvements to agricultural practices, how do we develop, test and put into practice new farming systems and tools? These are two important challenges that are being considered in the broader context of *sustainability*. In this chapter, sustainability is defined as *the ability to meet needs over an indefinite period of time*. As such, sustainability is a long-term goal, and our ability to achieve this goal depends on how effective we are in modifying essential



$$\text{Crop Production Effectiveness} = f(\text{efficiency, environmental condition, demand})$$

Figure 2.1 The relationship between crop production endpoints and the goal of sustainability.

activities like agriculture. Figure 2.1 depicts a simplified view of the relationships between crop production efficiency (i.e. outputs/inputs) and sustainability. An intermediate in this continuum is termed *crop production effectiveness*, which we use as one indicator of sustainability since it is a function of efficiency, environmental impact and consumer demand. Assessing proposed crop production modifications for their fit with sustainability starts with quantitatively or qualitatively measuring changes to those components that are clearly related to something of value or a condition one wishes to attain. These essential components, termed *endpoints*, include inputs, outputs, measures of environmental condition including susceptibility to erosion and habitat suitability for a diversity of organisms (i.e. biodiversity) and measures of consumer demand like nutritional quality and taste. The fact that effective crop production is more a case of optimizing rather than maximizing production reflects the qualitative, value-laden nature of the relationship between endpoints.

Genetic modification of plants through biotechnology is being developed and carefully assessed for its fit in specific production systems. The adoption rates in areas where these crops have received regulatory clearance have been dramatic, and experience to date has been that the visible environmental impacts are minimal (James, 2003). When evaluating the changes to the effectiveness of crop production

environmental systems that include GM crops, the key environmental assessment question becomes: does the use of GM crops mean that the practice is more effective than those it is replacing or augmenting? On the basis of evidence collected to date comparing the GM crops currently available to their traditional counterparts in production systems, there is evidence to support the assertion that many of these new crops are improving the effectiveness of industrial agricultural practices by increasing efficiency and reducing negative impacts to agricultural biodiversity. Specifically, one class of GM crops seems to have reduced inputs by reducing tillage operations and pesticide applications compared to traditional, non-GM crops (Fawcett & Towery, 2002). This chapter is intended to present a perspective on the 'state of play' of GM crops by examining information about the current applications of GM crops. Some insights into future opportunities using GM technology are also presented. This chapter is not intended to paint a 'perfect world' scenario for any technology, since that is not useful. Rather, it is hoped that the arguments supporting the development and use of GM crops are presented constructively, in a manner that encourages constructive dialogue rather than catalysing divisive debate (Nickson, 2003).

2.2 A need for better tools in crop production systems

2.2.1 Crop production and sustainability

The origins of crop production date back approximately 10 000 years when humans began to actively manage food production by cultivating plants on areas of land dedicated for this purpose. Over the years, people have selected crops and plant phenotypes on the basis of a number of criteria that included yield, ease of production, taste and visual appeal, storage stability, processing qualities, feed performance, demand created by trading partners and other characteristics. The consequences of these choices have been far-reaching for the human population, the society and the environment. First, population growth rates have increased dramatically, concurrent with the evolution of efficient crop production (Buhr & Sinclair, 1998). Second, managing the production of crops has enabled humans to develop social systems around villages and towns and to engage in trade, and has freed a portion of the community to pursue other occupations such as crafts, trading and arts. Third, the environmental impact of this coevolution has been dramatic in terms of environmental change. Old growth forests were transformed by logging, fields were created on alluvial plains as a result of controlling seasonal flooding, and prairies and certain processes that maintained them (i.e. seasonal fires) were changed to allow agricultural fields to prosper. As such, plant production practices have coevolved with social, economic and ecological systems; each has been effecting changes to the other for millennia.

Looking more closely at changes to the environment, as landscapes were transformed from unmanaged to managed for a chosen crop, two things have happened:

new ecological niches have been created and the changes in management practices have set in motion a new dynamic between environmental conditions and crop production goals. First, committing land to the production of a limited number of specific plants afforded an opportunity for certain organisms to thrive. Some of these organisms that moved in to occupy the niche reduced the quantity, quality and marketability of the harvested product. These were considered pests, and farmers have developed many ingenious ways to manage them over the years. Second, a dynamic situation arose because of this purposeful conversion to a biologically unnatural condition¹ combined with the desire to maximize the yield. As farmers combated, and in some cases defeated pests, new pests arrived and/or the former pests adapted to the new conditions. With each adjustment, the field environmental conditions changed in terms of the composition and abundance of species and communities (biodiversity), as well as soil and water quality. The cropping systems, as they exist today, are the result of choices people made to produce something of value – sufficient, good-tasting food, safe living conditions and an improved quality of life.

There are three general descriptions used to characterize agricultural systems today: intensive or industrial, subsistence and transition (McCloud, 1998; Raven *et al.*, 1998). Industrial crop production systems require fewer farmers to manage larger tracks of land and to produce a narrow diversity of crops than does subsistence farming. Industrial agriculture requires inputs derived from fossil fuels, and is widely believed to have greater impacts on biodiversity than does subsistence farming (McCloud, 1998; Raven *et al.*, 1998). Because industrial systems are capital-intensive, they are more often found in economically developed countries. Subsistence farming uses energy primarily derived from humans and animals, and traditional knowledge to produce a greater variety of crops on small plots. The output feeds fewer people per hectare than does an industrial system. Economies in transition tend to have production systems in transition. As such, farmers with more available money tend to purchase fossil fuel-based inputs to increase their efficiency and economic returns.

Successful transition farmers have more time and capabilities to plant larger areas, which requires access to land that may only be available by converting natural areas. However, natural areas are often not suited for long-term crop production, and they quickly degrade in terms of productivity and value. This highlights a fundamental issue involving an increasing population putting more stress on this limited land resource. Around 1830, the world population reached 1 billion, and doubled from 2.5 to 5 billion between 1950 and 1990. Population growth continues to accelerate as evidenced by the fact that the global population now exceeds 6 billion. As such, land and water are being used and degraded at dangerously high rates: ‘In 1950, some 115 million km² of the earth’s surface were undegraded, vegetated land. Just 40 years later, almost 9 million km², an area as large as China, were classified as “moderately degraded”, with greatly reduced agricultural productivity. A further 3 million km²

¹ Crop production based on the purposeful planting of selected plants within a chosen area is considered biologically unnatural.

were “severely degraded”, having lost almost completely their original biotic functions’ (FAO, 1996).

One of the important changes that occurred in the twentieth century was an awakening among the general public to the condition and importance of the environment. This greater awareness, recognized as an environmental ethic, has led various factions of society and academic disciplines to propose models for the future sustainability of the earth. The result is numerous publications conveying widely divergent opinions on what aspect of the environment should be sustained, why it is important, and how far we should be looking into the future (McIsaac, 1994). A subset of this broad subject is sustainable agriculture and crop production, which is equally important and relevant to environmental management. Sustainable agriculture is defined here as ‘the agricultural system’s ability to meet needs over an indefinite period of time’. A more functional definition requires detailed knowledge of the needs that must be met by the agricultural system, including cultural, economic and environmental needs. From a purely scientific view,² the basic elements of this model are beyond our current level of understanding (Power, 1994). Furthermore, there are no historical states to serve as appropriate references for sustainable agriculture systems. Some authors have proposed using natural ecosystems as models for sustainability, but these are not relevant to the central purpose of an agricultural system that has evolved over thousands of years: to provide food, feed and fibre for current and future generations (Power, 1994). As such, sustainable agriculture is better considered as a description of an ideal state that has no perfect point of reference in nature.

Developing solutions to problems such as the need to improve the sustainability of crop production requires that we recognize and understand the current farm practices, their interrelatedness and the nature of their environmental impacts (Figure 2.1). Cropping system characteristics that are generally considered as important in terms of sustainability include production efficiency (level of inputs such as fossil fuels and generation of unusable waste), impacts on soil quality, impacts on biodiversity and the genetic attributes of the seed used that can affect efficiency and consumer demand (nutrition, flavour, colour, etc.). Current crop management regimes have benefited humans by meeting demand for foods, animal feeds and fibres. Improvements in efficiency and reductions in environmental impacts have also been achieved. Nevertheless, some practices that are accepted and widely used today are in need of improvement. For example, plowing is widely employed to control weeds, but it disrupts the soil community, facilitates the loss of moisture due to exposure to air and leads to erosion of topsoil.

It can equally be argued that the application of prophylactic chemicals for crop protection has benefited society by enabling farmers to control the many pests that

² As noted, there is no consensus definition of *sustainability*, but McIsaac (1994) believes there are some common points that include cultural values and other non-scientific points. In this chapter, we focus only on aspects that can be assessed scientifically, but acknowledge that sustainability encompasses much more.

reduce yield and affect product quality. However, residues of these chemicals in soil and water that run off cultivated fields can pass into associated watercourses and so affect aquatic ecosystems. Other crop production management practices and tools can affect biodiversity inside and outside the field by degrading or polluting the physical or chemical environment. The application of non-specific crop protection chemicals can decrease the abundance and diversity of organisms within the field and also in adjacent habitats as a result of spray drift.

There are two key inputs essential to the effectiveness of crop production that also affect the sustainability of industrial agriculture: fossil fuels and crop genetics. Improvements in efficiency of crop production have been made possible by thorough use of significant quantities of fossil fuels required to perform tillage operations and pesticide applications. Fossil fuels are also used in the manufacture and transport of pesticides and fertilizers, and the containers used to transport pesticides are often non-renewable, requiring disposal. However, increasing use of fossil fuels is not sustainable and so changes are needed to the volume of fuel used and to its patterns of use.

Crop genetics also impacts the input and output contributions to production efficiency. Seeds of most major crops have been selected over the years to have uniform characteristics for emergence, plant height, yield and increased resistance to pests and diseases. Genetics has been an essential tool for plant breeders to improve cropping systems over the last 150 years. We have not realized the full potential of genetics as a tool for improving the effectiveness of crop production systems.

Finding practical and timely solutions to the current problems necessitates thinking of sustainability as a goal, and examining new crop production practices and technologies through the question, 'are the changes we intend to introduce *more sustainable* than the current procedures?' An assessment approach based on agricultural sustainability can embrace the environmental ethic of the twenty-first century, while also recognizing the fact that cropping systems evolve with new knowledge and cultures.

If the goal is to develop more sustainable agriculture, then it is appropriate to compare new technologies with the existing ones. The necessary elements of the comparison, *assessment endpoints*, must be linked to environmental properties that are measurable by scientific methods and meaningful in terms of sustainability. In this way, assessment of GM crops sharing a common set of traits (e.g. herbicide tolerance or pest resistance) would be based on the view that creating more effective production systems could increase agricultural sustainability. Traits that increase efficiency and facilitate using less ecologically disruptive production practices would be judged to be more effective and ultimately more sustainable (Figure 2.1). Furthermore, it should be borne in mind that the employment of GM crops represents only one potentially valuable tool towards achieving a goal of more effective crop production. Other tools must also be considered, developed and assessed with the same rigour as is applied for the use of GM crops.

Table 2.1 Countries where GM crops have been commercialized for planting as of 2003¹

Country	Area planted (million hectares)	Global total (%)
United States	42.8	63
Argentina	13.9	21
Canada	4.4	6
Brazil	3	4
China	2.8	4
South Africa	0.4	1
Australia, Bulgaria, Colombia, Germany, Honduras, India, Indonesia, Mexico, Philippines, Romania, Spain, Uruguay	Each with <0.1	<1

¹ James, 2003.

2.3 The current state of GM crops

For the past 8 years, the International Service for Acquisition of Agri-biotech Applications³ (ISAAA) has collected information from technology providers and other credible sources to publish 'Global Status of Commercialized Transgenic Crops' on a yearly basis. The most recent publication notes that, as of 2003, 18 countries have approved one or more GM crops for commercial planting (intentional release) (James, 2003). The estimated total area planted to GM crops was 67.7 million hectares, an increase of approximately 15% from 2002, when the global area planted was over 58 million hectares (James, 2002). As depicted in Table 2.1, the vast majority of plantings of GM crops occurred in the countries that were the earliest adopters of the technology and within industrial crop production systems. Argentina, Canada and the United States had all approved at least one GM crop by 1996, the year ISAAA began collecting these commercialization data. Interestingly, the rate of adoption, as measured by area planted to GM crops, has been fairly steady at > 10% in both developed and developing countries since 2000 (Figure 2.2). When viewed from the perspective of adoption by farmers, the change in hectares from 2002 to 2003 reflects an approximate increase of 1 million growers (>16%) who are now choosing to grow GM crops over their non-GM counterparts. According to James (2003), 7 million farmers in 18 countries planted GM crops in 2003, whereas an estimated 6 million growers in 16 countries were recorded in 2002.

To date, a narrow range of GM crops are included amongst those planted in the countries listed in Table 2.1, namely, soybean, maize, cotton and canola (oilseed rape). Some authors have noted that this list is not reflective of the potential diversity of crops that can be genetically modified (Dunwell, 2000). Also absent from this list are certain products that have been approved and perhaps marketed in the past. Products like delayed ripening tomatoes (Flavr Savr[®]), insect-protected and

³ ISAAA is a not-for-profit public charity working to alleviate poverty in developing countries by facilitating the transfer and sharing of crop biotechnology applications to increase crop production and income generation, particularly for resource-poor farmers, and to bring about a safe environment and more sustainable agricultural development (www.isaaa.org).

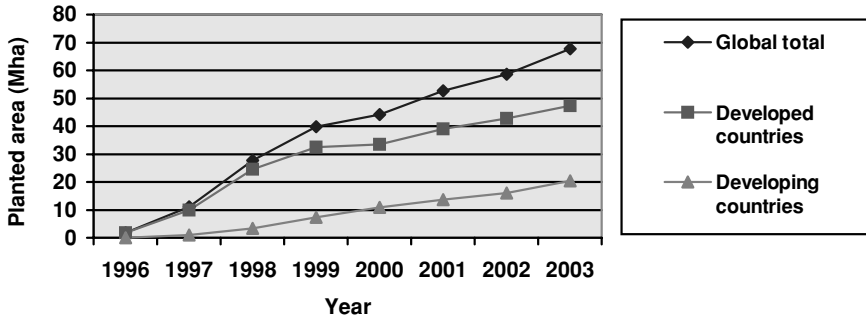


Figure 2.2 Planting area of GM crops and rate of growth. Values for hectares in developing and developed countries from 1998 to 2001 are estimated from James, 2003.

insect-and-virus-protected potatoes (NewLeaf[®] and NewLeaf+[®]), virus-resistant squash and tomatoes with improved processing characteristics (lower water content) have all been approved for planting in at least one country. Because of market and competitive forces, however, these products are no longer commercially available or they are only being used on a very limited scale (<1000 ha).

Some confusion exists today because the number of products that have completed regulatory review process is much greater than the number of GM crops that are actually available commercially. Recently, the US government launched a new Unified Biotech Web site (see <http://usbiotechreg.nbii.gov/>) containing a searchable database for GM plant products that have completed the necessary reviews for food, feed or planting in the United States. The new database was developed to coordinate information on approvals in the United States for the Biosafety Clearing House (BCH) under the Cartagena Protocol for Biosafety. It is intended to be a comprehensive listing of every GM event⁴ that has completed regulatory review. However, many of these materials are not commercially available for various reasons. As noted earlier, products like Flavr Savr[®] tomatoes and NewLeaf[®] (insect-protected) potatoes had been grown commercially, but were later discontinued because of market issues. In addition, a number of events have completed regulatory review, but were never released commercially for market reasons. Information on all GM crops can be found on the Unified Biotech Web site.

However, a problem with such a comprehensive database is the false impression it creates that more products are being grown commercially than is the case. To address this issue, the Biotechnology Industry Organization (BIO) is developing a Web site that states whether an event is commercialized or discontinued, and whether it was ever commercialized (Table 2.2). While this database is not available at present, the BIO Web site (<http://www.bio.org/foodag/agbiotechprod.asp>)

⁴ The term *event* is used to designate a unique transformation event, e.g. MON810 is a unique maize transformation event containing a *cry1Ab* gene, and as such has undergone a regulatory approval process.

Table 2.2 List of GM crop products available for sale¹ as of February 5, 2004²

Product	Event(s)	Company
BXN [®] Canola	OXY-235	Bayer CropScience, LP
LibertyLink [®] Canola	T45	
	Topas 19/2	
SeedLink [®] Canola	MS1/RF1	
(a male sterility/fertility restoration system used in hybrid canola seed production)	MS1/RF2	
	MS8/RF3	
LibertyLink [®] Maize	T25	
LibertyLink [®] Cotton	LLCotton25	
Herculex [™] I (maize)	1507	Dow AgroSciences, LLC
Roundup Ready [®] Canola	RT73	Monsanto Company
Roundup Ready [®] Corn	GA21	
	NK603	
Roundup Ready [®] Cotton	1445	
Roundup Ready [®] Soybean	40-3-2	
YieldGard [®] Corn Borer Corn	MON810	
YieldGard [®] Rootworm Corn	MON863	
Bollgard [®] Cotton	531	
	757	
Bollgard II [®] Cotton	MON15985	
Pioneer Brand Seed Corn with Herculex [™] I	TC1507	Pioneer Hi-Bred International, Inc. (a DuPont Company)
Attribute [®] Sweet Corn	BT11	Syngenta Seeds, Inc.
KnockOut [®] Insect Resistant Corn	176	
NK brand GM Bt corn with YieldGard [®]	BT11	

¹ Each product is being sold for commercial use in one or more countries. A Bt cotton event is commercially available in China. Events GK and sGK have been used in China since 1997 and 1999, respectively (Shelton *et al.*, 2002). In addition, a virus-resistant squash and papaya (55-1) are commercially available. Papaya seeds/plantlets are not sold commercially. These products are not listed on the BIO Web site.

² Available at the BIO Web site.

provides a list of products that are currently commercialized. Between the US Unified Biotech and BIO Web sites, one can obtain accurate information concerning both GM plant approvals and products. Recently, Nap *et al.* (2003) and Dunwell (2000) have highlighted the fact that there are several other specialist Web-based database systems providing information on various groupings of GM crops (Table 2.3).

As of 2004, two traits have dominated among the commercial GM crops: herbicide tolerance (HT) and insect protection (Bt) (James, 2003; Silvers *et al.*, 2003). Of these two traits, HT has been predominant in terms of area planted (James, 2003), and the specific HT traits currently available comprise of those conferring tolerance to three different active ingredients, glyphosate (Roundup Ready[®]), glufosinate (LibertyLink[®]) and bromoxynil (BXN[®]), in descending order of acreage.

Interestingly, not all HT products that are commercially available are defined globally as GM, and as such not all HT products undergo the same rigorous regulatory scrutiny. Because breeders have been able to develop a wide variety of crops that are tolerant to triazine, imidazolinone and sulfonylurea-based herbicides

Table 2.3 Additional information sources for GM crops

Organization	Information	Web address
Agriculture and Biotechnology Strategies Inc. (AGBIOS)	GM crops that have received regulatory approval for release	www.agbios.com
Biosafety Information Network and Advisory Service (BINAS)	Global field trials	www.binas.unido.org/binas/trials.php3
Information Systems for Biotechnology (ISB)	Field testing and petitions for deregulation	www.isb.vt.edu/cfdocs/fieldtests1.cfm
Organization for Economic Cooperation and Development (OECD)	Field testing	www.ois.oecd.org/biotrack.nsf
US Patent Office	Patent information	patents.uspto.gov/access/search-adv.html

through ‘traditional’ techniques, these products are not deemed to be GM cultivars. Only in Canada⁵ have conventionally bred HT crops recently undergone regulatory review and approval prior to commercialization. This chapter does not consider such plants and refers throughout only to GM HT material.

2.3.1 *Herbicide tolerance*

Growers, particularly in the developed world, have embraced HT technology as evidenced by its rapid adoption (James, 2003). According to James (2003), HT soybeans account for 55% of all soybeans grown globally. This is because Argentina, Brazil and the United States, three of the world’s largest producers of soybean, have collectively planted approximately 40 million hectares of HT soybeans. Some of the reasons given for the phenomenal growth of HT crops (canola, cotton, maize and soybean) in general have been grower-focused: ease of weed control, improved control, less crop injury, concordance with soil conservation practices, economic return and use of herbicides that are less toxic to humans and that degrade rapidly in the environment (Burnside, 1996; Culpepper & York, 1998; Roberts *et al.*, 1998; McKinley *et al.*, 1999; Carpenter & Gianessi, 2001a; Fawcett & Towery, 2002; Runge & Ryan, 2003).

The fact that each HT crop produced thus far is associated with a specific, broad-spectrum herbicide is an integral factor in determining the value and adoption of the product. In the case of Roundup Ready[®] (RR) crops, the active herbicidal agent is glyphosate, the properties of which are described by an extensive worldwide human health, safety and environmental database (Giesy *et al.*, 2000; Williams *et al.*, 2000). Glyphosate has been thoroughly reviewed and registered by the US Environmental Protection Agency and other regulatory agencies around the world (US EPA, 1993;

⁵ Canada regulates plants with novel traits (PNTs). See <http://www.inspection.gc.ca/english/plaveg/bio/pntchae.shtml>.

EU, 2002). Glyphosate has an excellent human health and environmental profile and a long history of safe use in more than 130 countries. When used according to label directions, glyphosate does not present any unreasonable risk to human health and the environment.

Nelson and Bullock (2003) examined existing data for herbicide use in soybean production systems in eight midwestern states in the United States to simulate the relative environmental effect of RR soybeans. The approach used is case-specific, relevant only to RR soybeans, but could be used for other HT crops that substitute one or several herbicides for a single, broad-spectrum weed management tool. Their analysis assumed either 100 or 0% adoption of RR soybean on over 1000 farms covering 145 371 ha. The authors justified their approach based on an earlier report (Bullock & Nitsi, 2001) where the profitability of the RR weed management system in soybean was shown to be the key factor in its widespread adoption. Nelson and Bullock (2003) selected the net toxicity of herbicide use per acre as an indicator of environmental impact, since manufacturers must have rat LD₅₀⁶ data available on the active herbicidal agent. Their argument that toxicity per acre is more relevant to assessing environmental impact than weight or volume of applied chemical is supported by earlier work on environmental indicators (Nelson & Miranowski, 1996). In their conclusion, Nelson and Bullock (2003) stated that '[RR] soybean seed technology is more environmentally friendly than non-[RR] technology for all farms in the dimension of acute mammalian toxicity'. Furthermore, their approach highlights the dubious relevance of the simpler 'pounds on the ground' analyses to assess environmental impact of HT crops since the herbicides differ in their environmental properties.

The results of Nelson and Bullock (2003) and experience with HT crops support the position that they increase the effectiveness of crop production systems. While there is reason to be optimistic, it is just as important to remain watchful since beneficial technologies like HT crops must be carefully managed to maintain their value. In this vane, while growers have been very quick to adopt HT in soybean, maize, cotton and canola, the rapid rate of adoption has caused others to urge vigilance (Reddy, 2001; Martinez-Ghersa *et al.*, 2003; Beckie *et al.*, 2004). Stewardship is not an issue of GM *per se*, but it is an integral part of any cropping/pest management system. Concerns about the potential for developing weeds resistant to herbicides are an important commercial issue requiring stewardship at all levels of the weed-control system (growers, applicators, technology providers, etc.). The probability that resistance will develop among weeds as a consequence of more frequent exposure is largely a function of the herbicide and its mode of action, with development of resistance to some herbicides like glyphosate having been slower than for other classes like ALS inhibitors⁷ (Jasieniuk, 1995). Nevertheless, stewardship is an important component of product use recommendations for herbicides. Because the herbicides associated with GM crops are integral to the value of the

⁶ LD₅₀ is a dose that effects lethality in 50% of a test group.

⁷ ALS inhibitors are a class of herbicides that inhibit acetolactate synthase, an enzyme involved in the production of certain aliphatic, essential amino acids in plants.

product, there is a desire to steward all products (Reddy, 2001), especially those that have favourable environmental properties.

2.3.2 *Insect protection*

The second predominant trait available in commercial GM crops is insect protection, based on several related proteins originally isolated from *Bacillus thuringiensis* (Bt) (Schnepf *et al.*, 1998). The *Bt* or *cry* genes currently available in GM crops represent only a small fraction of those that have been identified in *B. thuringiensis* (Lereclus *et al.*, 1993; Crickmore *et al.*, 1998). Many of the proteins encoded by other genes in this gene family also possess insecticidal activity (Lereclus *et al.*, 1993). Insect protection in current GM crops is derived from one to three well-characterized *Bt* genes inserted into specifically chosen crops. The Bt protein(s) produced *in planta* confers protection against insect pests with markedly increased specificity compared to broad-spectrum insecticides currently being used in agricultural production systems. To date, seven discrete *cry* genes have been inserted singly or in combination with a second *cry* gene into maize, cotton and potato and commercialized (Table 2.2), though Bt potatoes are no longer being marketed (Silvers *et al.*, 2003).

One of the most attractive features of Bt crops is their well-known specificity compared to broad-spectrum insecticides (Frankenhuyzen, 1993; Shelton *et al.*, 2002). For example, Cry1 proteins are active only against Lepidopterans (caterpillars) whereas Cry3 proteins target coleopteran species (beetles) (Frankenhuyzen, 1993). However, the insecticidal activity is much more specific within the taxonomic orders for the individual Bt proteins, with some genera exhibiting more susceptibility than others within a family. Within families, different insect pests will display different levels of sensitivity to the Bt protein expressed in the crop. The US Environmental Protection Agency (EPA) Scientific Advisory Panel (SAP) examined the issue of susceptibility of individual species to Bt proteins in 1998. They developed three classifications for pests, which are 'targeted', 'non-targeted directly affected' and 'non-targeted indirectly affected'. Targeted pests, according to the US EPA SAP (1998), are those most susceptible. Table 2.4 lists the targeted pests for the GM Bt crops discussed by SAP in 1998. Highlighted in their analysis is that targeted pests will change depending on the region of the world where the GM Bt crop is grown.

Selection of a *cry* gene for insertion into a crop is based on the sensitivity of the insect pest to the given Bt protein. After transformation of the crop, the final product is selected based in part on the efficacy of the GM crop under high insect pest pressure under a variety of environmental conditions (Appendix 2 of the US-Canada Agreement, n.d.). However, control of other closely related insects that may be less susceptible or affected by changes in pest management as a result of using the GM Bt crop is also carefully studied. For example, because GM Bt crops are known to reduce the amounts of insecticides used (Heimlich *et al.*, 2000; Carpenter & Gianessi, 2001b; Kalaitzandonakes & Suntornpithug, 2001; US EPA, 2001; Shelton *et al.*, 2002), changes to the insecticide use can result in changes to the population dynamics of

Table 2.4 List of targeted pests for three GM Bt crops developed by the US EPA SAP (1998)

GM Bt crop	Pest designation	Targeted pest description
Bt maize (Cry1Ab, a lepidopteran active protein)	Target pest widely present in the United States	European Corn Borer (ECB), <i>Ostrinia nubilalis</i> (Hubner); Southwestern Corn Borer (SWCB), <i>Diatraea grandiosella</i> (Dyar)
Bt cotton (Cry1Ac, a lepidopteran active protein)	Target pest in the mid-south and southeast	Tobacco Budworm (TBW), <i>Heliothis virescens</i> (Fabricius); Cotton Bollworm (CBW), <i>Helicoverpa zea</i> (Boddie)
Bt cotton (Cry1Ac, a lepidopteran active protein)	Target pest in the southwest	Pink Bollworm (PBW), <i>Pectinophora gossypiella</i> (Saunders); Cotton Bollworm (CBW), <i>Helicoverpa zea</i> (Boddie); Tobacco Budworm (TBW), <i>Heliothis virescens</i> (Fabricius)

other pests. These changes may give rise to other non-targeted pests that must be examined in the course of assessing the value of a GM Bt crop.

In addition to the Bt products based on control of caterpillars with Cry1A and Cry1F proteins, a new maize product was recently marketed in the United States that provides protection against corn rootworm (*Diabrotica* spp.). The product, MON863 (Table 2.2), expresses a Cry3Bb1 protein that is active against specific Coleopteran pests of the genus *Diabrotica*, which are known to cause severe economic losses in the United States and, more recently, within the EU. Current control is based on a combination of crop rotation and a selection of insecticides, but the future success of these practices is uncertain (Rice, 2003). Certain biotypes of two species of corn rootworm have adapted to rotation approaches, making insecticide treatment the only practical means of control (Chiang, 1965; Krysan *et al.*, 1984). In parts of the US Corn Belt, adults of one species of rootworm have developed resistance to foliar-applied insecticides (Meinke *et al.*, 1998). Rice (2003) has assessed the potential value of MON863 maize in terms of insect control, economic return along with other important benefits to growers and the environment, and concluded that the use of transgenic rootworm maize 'has the potential to transform integrated pest management efforts dramatically in the Corn Belt'.

One of the primary concerns associated with the use of any pesticide is the development of resistance to the active agent by the target pest. This concern is particularly acute for products like GM Bt maize and GM Bt cotton because of their value to growers and the environment through reduced use of chemical insecticides (Carpenter *et al.*, 2002). Technology providers have directed much thought and resources to work with government and academic scientists to develop insect resistance management (IRM) strategies that are scientifically based yet sufficiently practical to ensure implementation by growers (US EPA, 2001; Tabashnik *et al.*,

2003; Carrière *et al.*, 2004). The GM Bt crops available today in the United States must have an IRM plan approved by regulatory authorities prior to consent to market being granted by the EPA.

Technology providers are required to implement a comprehensive IRM programme for each commercial GM Bt crop (US EPA, 2001). The goal of this programme is to maintain the susceptibility of targeted insects in order to sustain GM Bt crop performance and the associated economic and environmental benefits. Key components of the IRM programme target the technology provider's responsibilities for risk management and risk mitigation, while other components focus on the grower's responsibilities to understand and adhere to IRM requirements. The essential elements of the IRM plan include planting 'refuges' to sustain pest susceptibility, educating growers of their IRM plan responsibilities, monitoring levels of susceptibility to a specific Cry protein in the target pest using laboratory bioassays and identifying options to mitigate the spread of resistance should it develop. The latter may include treatment with an approved insecticide if the levels exceed a threshold and continued monitoring of field collected specimens for their susceptibility to the Bt protein. In addition, grower compliance to the refuge requirements is monitored. Growers that continually fail to implement the IRM plan requirements may be denied access to GM Bt crop technology. As such, implementing an effective IRM plan relies upon collaboration and commitment from a variety of experts, including growers, agricultural specialists and academic and industry scientists, working together to ensure that GM Bt crop performance is sustained. To date, no incidence of resistant insects has been detected because of exposure to GM Bt crops in the field despite the widespread adoption and expansion of GM Bt crop cultivation since 1996 (Tabashnik *et al.*, 2003). However, agricultural experts have learned that vigilance is essential. Experience with chemical insecticides and microbial Bt products teaches us that appropriate and thorough IRM is key to the durability and value of GM Bt crops (Carrière *et al.*, 2004). The current programme used by industry and supported by academic and government scientists appears to be working well, although it will necessarily be modified based on new knowledge and experience with GM Bt crops.

Currently, the reasons growers have given for using GM Bt crops have varied depending on the crop and the pest (Shelton *et al.*, 2002). Maize growers in the United States use Cry1Ab-expressing maize to protect against damage from European corn borers (*Ostrinia nubilalis*) (Table 2.4). Compared to other insect pests in maize, the introduction of Cry1Ab-protected maize has resulted in a modest but significant decrease in insecticide applications (Heimlich *et al.*, 2000; Carpenter & Gianessi, 2001a; Pilcher *et al.*, 2002). Conversely, because of the extensive use of insecticides used in cotton to control bollworms (*Helicoverpa zea*) and budworms (*Heliothis virescens*), adoption of Cry1Ac and Cry1Ac stacked with Cry2Ab cotton has resulted in a significant decrease in insecticide use (Carpenter & Gianessi, 2001a; James, 2003; Qaim & Zilberman, 2003). Because of the human toxicity associated with many insecticides used in cotton production, GM Bt maize and GM Bt cotton have shown an excellent solution (Qaim & Zilberman, 2003; Rice, 2003) especially in

developing countries where small-holder farmers predominate (Qaim & Zilberman, 2003).

Secondary benefits of having Bt genes in maize and cotton have been noted in the marked reduction in levels of mycotoxins in the harvested grain (Munkvold *et al.*, 1999), reduced incidence of certain diseases associated with insect damage (Rice, 2003) and perhaps in reducing losses due to storage pests (Sedlacek *et al.*, 2001). Improving the quantity and quality of grain harvested would have significant health benefits in developing world areas, where a greater proportion of maize is used directly as a human food.

In summary, the information and experience with GM Bt crops appears to fit the description of a more effective crop production system than equivalent systems with non-GM crops. Their deployment reduces the need for certain inputs that are derived from fossil fuels (pesticides and pesticide applications) and have been shown to improve the quality of the harvested grain by indirectly reducing the levels of mycotoxins. As such, inputs are reduced without a cost to yield, biodiversity, soil quality, nutrition or other important characteristics.

2.3.3 *Virus resistance in plants*

As noted earlier, several other traits have been introduced, field-tested and commercialized in GM crops. A third agronomic trait is resistance to viruses, which was first demonstrated by inserting a gene derived from the pathogen (tobacco mosaic virus) itself (Powell *et al.*, 1986). Since that first development, several other mechanisms of pathogen-derived resistance have been studied and tested in GM plants (Beachy, 1997). Despite the fact that only two crops are protected from viral disease today, and the acreage is modest, virus-resistant GM plants offer exciting potential to aid farmers in their struggle to produce food (Gianessi *et al.*, 2002; Tepfer, 2002; Silvers *et al.*, 2003). Evidence of this potential and opportunity is found in the number of field trials being conducted in the United States with GM plants containing virus-resistant traits. Tepfer (2002) reported that, as of October 2001, 794 approvals had been granted for US field trials of GM viral-resistant plants covering a wide diversity of crops.

By 2001, three crops, squash, potato and papaya, containing genes derived from pathogenic viruses had been deregulated in the United States. Today, virus-resistant papaya and squash remain on the market. Virus-resistant squash and potato had a low adoption rate, probably because of many factors including consumer acceptance (Silvers *et al.*, 2003). Nevertheless, papaya resistant to ringspot virus is a good example of how a technological innovation possibly saved an industry in a region (Gonsalves, 2003). This product was being developed at the University of Hawaii and the US Department of Agriculture (USDA) at the same time as papaya ringspot virus (PRSV) was spreading throughout the Hawaiian production fields (Gonsalves, 1998, 2003). Interestingly, the first field trial with GM papaya was launched 1 month before the PRSV was discovered in the major production area in 1992. Within 5 years, a GM papaya (55-1) that demonstrated commercial levels of resistance

to PRSV was developed and approved (Lius *et al.*, 1997). Coincidentally, by 1998, within 6 years of first detecting the virus in the major producing area, production of papaya was reduced by over half in Hawaii (Gonsalves, 2003). Resistance to PRSV was so effective that growers were able to reclaim abandoned orchards and re-establish production fields. Today, the papaya production in Hawaii has recovered significantly in terms of yield, with no evidence of resistance breaking down. As with any other pest resistance trait, vigilance and stewardship of the technology will be necessary to ensure long-term utility of the trait. There is widespread recognition of the need to continue to monitor the resistance to PRSV in Hawaii and to prevent the introduction of exotic strains of the virus (Gonsalves, 2003). In this case, the contribution to crop production effectiveness has been critical to the survival of the papaya industry in Hawaii.

2.4 Future developments

The preceding discussion described a limited number of GM crop products that are currently available as commercial products (Table 2.2). In total, there are 21 GM crop products derived from six crops (canola, maize, cotton, papaya, soybean and squash) and four traits (HT, Bt, virus resistance and male sterility/fertility restoration) available to certain growers⁸ today. This portfolio has a narrow diversity when compared to the number of plants that can be transformed using recombinant DNA techniques (Dunwell, 2000; Babu *et al.*, 2003; Silvers *et al.*, 2003). Other agriculturally significant plants such as wheat, rice, alfalfa, sweet potato, cassava and several species of trees have been successfully transformed and field-tested around the world. Their importance to food security and economic development makes these and other plants key targets for improvement using GM techniques. In this section we will describe the future GM crops in the context of their potential impacts to production effectiveness and the sustainability of production agriculture.

The following discussion examines future developments in new traits and their likely impact on the input or output contributions to efficiency. Traits like HT, Bt and virus resistance are associated with reducing and substituting certain inputs in agricultural production systems. The first section describes the expansion and value-added uses of these familiar input traits. Secondly, traditional breeding has targeted improvements in crops to tolerate various abiotic stresses such as drought, salt and cold. Modern biotechnology affords the potential to aid plant breeders in achieving this goal, and some of these advances will be discussed. In addition, many GM crops are being tested and developed that have the potential to improve the output of food production, and hence increase the efficiency of the agricultural system. Advances in this area will be examined below. Lastly, gene flow and gene containment are relevant to the management and mitigation of potential risks associated with GM plants. Because this is an area of scientific interest and investigation, a description of some of the research in this area is presented.

⁸ These products are legally available to growers where they have completed regulatory review.

2.4.1 Expansion of Bt and HT

In the immediate future, expanded use of the Bt and HT GM crops will be a focus area for seed companies and technology providers. Both traditional breeding and modern biotechnology techniques are being used to combine traits (stacking) such as Bt with HT and with different variants of the Bt gene family. Because of the incremental value to growers, a strong inherent demand has developed for GM plant crops containing both Bt and HT. In response to this demand, crops such as maize and cotton stacked with Bt and HT are becoming available to growers or are undergoing regulatory review in many places around the world. The principle value offered by Bt stacked with HT is the additional benefits of the individual traits: simplified management of agricultural pests including weeds and specific families of insects and reduction in pesticide usage and tillage operations performed. Because of the value to growers, stacking of Bt and HT traits is an attractive commercial opportunity for technology providers. There is little interest at the present time to stack different HT traits for production agriculture systems.

Stacking different Bt traits provides value to growers by either controlling a broader range of insect pests or enhancing the likelihood of product durability, or both. For example, a Bt maize product (e.g. MON810) that controls corn borers (lepidopteran pests) can be crossed using conventional breeding techniques with another Bt maize (e.g. MON863) that controls corn rootworm (coleopteran pest). The resultant stacked product can be used to control more maize insect pests and eliminate or dramatically reduce the need for insecticides. One such product that combines control of corn rootworm with corn borer control is close to being marketed in the United States. The combination of Bt traits, Cry3Bb1 with Cry1Ab, will result in substantially improved insect pest management and reduced pesticide use in maize (Rice, 2003).

As noted above, the value and durability of GM Bt crops are protected over time through IRM. A more durable product is one that has a lower likelihood of resistance development in target insects. Cotton containing two Bt genes with different modes of action is now available to growers in the United States and Australia. This product, known as Bollgard II, utilizes *cry1Ac* and *cry2Ab* genes to control a broader spectrum of Lepidopteran pests and to provide better IRM in the primary target insects (Moar, 2003). Using two genes (pyramiding) with different modes of action along with a refuge (fields planted to non-Bt cotton to reduce the selection for resistance alleles in the pest population) will likely result in a highly durable product (Moar, 2003; Carrière *et al.*, 2004). Other similar examples of gene stacking to enhance product durability and agronomic value are also being developed for maize.

Because of the established safety record, simplicity and specificity afforded by Bt-based GM crops in controlling insect pests, field trials with various *Bt* genes in a number of different crops have occurred in several countries around the world. Stewart *et al.* (1996) prepared and tested a GM Bt canola (*Brassica napus* L.) in field experiments to investigate the potential fitness effect of a *cry1Ac* gene (Stewart *et al.*, 1997). However, this material was developed for research, and has not advanced

towards a market application to date. Others are examining the use of Bt genes in rice (*Oryza sativa*), alfalfa (*Medicago sativa*) soybean (*Glycine max*), eggplant (*Solanum melongena*), peanut (*Arachis* sp.) and various species of trees (Babu *et al.*, 2003; Silvers *et al.*, 2003) with the potential for becoming commercial products in the future. GM Bt rice has been reported to be an attractive possibility for use by resource poor farmers in China (Shu *et al.*, 2000), and has been field-tested (Ye *et al.*, 2001). In addition to increasing yields, GM Bt rice has the added benefit of reducing the exposure of growers and their families to toxic insecticides (Bennett *et al.*, 2003; Huang *et al.*, 2003) as noted by Rice (2003), in association with rootworm-protected maize. The growing interest in Bt-based products is not surprising given the success with GM Bt cotton (James, 2001), which has been approved in South Africa (Bennet *et al.*, 2003) and China (Huang *et al.*, 2003) since 1997, and recently approved for commercial use in India (James, 2003; Qaim & Zilberman, 2003; Jayaraman, 2002). As noted above, the key to the long-term durability and value of new GM Bt crops will be the quality of the IRM plan (Tabashnik *et al.*, 2003; Carrière *et al.*, 2004). Knowledge of the plant/pest complexes and feeding patterns of the pests (alternate hosts) and education of growers combined with experience based on the current GM Bt crops will be useful in designing effective IRM plans for future GM Bt crops. Similarly, stacking two or more Bt genes with different modes of action in these crops will likely be an important component of IRM and product efficacy.

The impact on crop production effectiveness can be demonstrated only after experience is gained with commercial use. It is logical to infer that there will be incremental gains made in IRM with products like Bollgard II® a new insect-protected cotton expressing Cry1Ac and Cry2Ab proteins, that will enhance the product's durability and further reduction in tillage operations by stacking Bt traits with HT traits.

2.4.2 Other pest resistance traits

Dunwell (2000) and others (Tepfer, 2002; Babu *et al.*, 2003) have highlighted that a wide variety of genes and crops are under investigation for potential market release. Different, non-*cry* gene based, insect protection technologies are under development including Vip3A, ToxinA, avidin, protease inhibitors and others (Babu *et al.*, 2003; Lee *et al.*, 2003; Liu *et al.*, 2003). Another technological innovation being investigated is the use of chimeric genes (Babu *et al.*, 2003) for their potential to confer more durable protection to the significant insect pests that limit crop yields. In the future, new genes or genetic mechanisms will be introduced into crops to provide selective and more durable protection against specific pests. Another exciting opportunity is the potential added benefit to the food production system resulting from a material with improved quality characteristics at harvest, e.g. lower mycotoxins and reduced weed seed in the grain. These traits will expand the current portfolio that is based on Bt, HT and particularly virus resistance genes whose true potential have yet to be fully realized (Tepfer, 2002).

2.4.3 *Tolerance to abiotic stress*

It is well known that abiotic stressors pose a significant environmental challenge to farmers around the world (Boyer, 1982). A second area for improvement of crop production efficiency is therefore enhanced tolerance to abiotic stresses such as drought, salt and water (Kasuga *et al.*, 1999; Dunwell, 2000; Bartels, 2001; Zhu, 2001; Wang *et al.*, 2003). Advances in genomics and other areas of research are now providing plant scientists with a better understanding of how plants react to salt, drought, heat stress, heavy metals and other abiotic factors. Genes are being discovered and tested in many plant species that could enable the crop to sustain high yield even under adverse environmental conditions. Such products, once they are developed and carefully assessed, have the potential to increase both production efficiency and perhaps cropping system diversity by providing opportunities to produce crops that are currently not adapted to the specific production environments. For example, growers in regions with low or variable rainfall must grow naturally drought-tolerant crops or utilize irrigation. The availability of additional drought-tolerant crops would increase the options available to such producers, and thereby allow them to better manage their economic risks. In addition, crops that produce economic returns on marginal land should reduce the pressure to convert more fertile, productive natural habitats into agricultural fields. As such, GM is enabling scientists to apply new information from genomics and other disciplines to key problems of food security and sustainability. There are no such products currently on the market, but it is known that the field testing is underway and success may be a few years away after careful evaluation and regulatory review.

2.4.4 *Output traits*

Crop production efficiency is a function of both the inputs (pesticides, fertilizers, tillage, water, etc.) and outputs (Figure 2.1). While traits like Bt, HT and abiotic stress tolerance increase or maintain yield without the need for more inputs, other types of traits will increase the output of a production system by affecting the quality of the harvested product. For example, a commodity derived from a GM crop with improved nutritional quality or one that reduces the cost of manufacturing a processed food increases overall crop production efficiency from the perspective of food production. In this case, the primary GM crop could impact the efficiency and environmental impact associated with the processing operation of food or feed product. Much research is underway in the area of output traits that impact efficiency by improving the characteristics of the harvested article in relationship to its downstream use. Examples that are discussed below include elevating the levels of essential nutrients in foods and feeds, reducing the levels of naturally occurring anti-nutrients and allergens and modifying the composition of a basic commodity that enables more efficient processing into a final product. A few recent publications provide an extensive review of these types of products (Mackey & Fuchs, 2002; Cockburn, 2004; ILSI, 2004).

Traditional breeding has successfully modified the composition of many foods and feeds around the world by modifying existing, toxic or nutritionally deficient plants to varieties suitable for consumption (e.g. tomatoes and potatoes). One example is the development of maize with increased levels of the essential amino acid lysine known as quality protein maize (QPM) (Bjarnason & Vasal, 1992). This maize is considered an important food in developing nations where lysine is often limiting in diets. There are several problems associated with QPM including susceptibility to diseases and certain storage pests. Using GM, maize with enhanced levels of lysine has been developed and is nearing commercialization in the United States and Argentina (O'Quinn *et al.*, 2000). Unlike QPM, enhanced lysine GM maize will not suffer disease susceptibility and storage problems. Enhanced lysine maize is currently being developed for use primarily as an animal feed to replace or significantly reduce the lysine that is added to diets, and would reduce the energy needed to produce lysine from fermentation in addition to the cost of waste disposal from these operations. By the addition of a single gene (*cordapA*)⁹, lysine levels in maize can be increased without the need for additional inputs in production. Other similar GM-based approaches are being used to improve the levels of essential amino acids in other crops (Falco *et al.*, 1995). One could envisage that this technology could address an important food issue in the developing world once concerns of acceptance are addressed.

Other output traits under investigation include plant production of compounds with potential human health benefits and feeds that improve the efficiency of meat production. Concern over the health effects of trans-fatty acids in diets has created intense interest in developing biotechnology-based solutions (Murphy, 1996). Products such as high oleic acid oilseed rape and soybean (Kinney & Knowlton, 1998) and oilseeds with reduced levels of saturated fats (Liu & Brown, 1996) are being developed using GM and traditional breeding. Another exciting opportunity in the area of improved nutrition is the discovery by Potrykus and Beyer (Ye *et al.*, 2000; Potrykus, 2001) that insertion of genes from daffodils into rice can significantly increase the vitamin A content. While there are many technical and other hurdles to overcome, 'golden rice' has the potential to address night blindness, which is an important health issue in India, Africa and parts of Asia where the lack of animal-derived foods can result in vitamin A deficiency. Other biotechnology-based strategies are also being explored to increase the vitamin A content in rapeseed oil (Dhawan, 2001). Mackey and Fuchs (2002) provide a good review of these and other nutritionally enhanced products, which are highly desired to the public because of their perceived health benefits.

Crop diversity is considered a component of a more sustainable agriculture because it reduces the vulnerability to a widespread crop loss and provides farmers with more production options. However, the safety of certain foods restricts the broader use of some crops like peanuts, which are a good source of protein, but are

⁹ The gene *cordapA* encodes a dihydrodipicolinate synthase (DHDPS) enzyme that is less sensitive to feedback inhibition by lysine than the endogenous DHDPS (Karsten, 1997).

also highly allergenic. Canola or oilseed rape (*Brassica napus* L.) has been modified using traditional breeding to reduce the levels of erucic acid thereby making the oil suitable for human consumption (NRC–CNRC, 1992). But canola meal contains high levels of anti-nutrients including glucosinolates and other natural products that make it unacceptable as a source of proteins for humans. Researchers are using GM to try to remove the allergens or render them non-allergenic in food crops like peanuts, rice, soybean, wheat and potatoes (Tada *et al.*, 1996; Buchanan *et al.*, 1997; Kleiner, 2002; Rabjohn *et al.*, 2002). Similarly, a GM-based approach to reduce the levels of glucosinolates in rapeseed has been published (Vageeshbabu & Chopra, 1997).

Demand for animal protein in diets is increasing globally as incomes rise. Depending on the species of animal, the efficiency of producing protein in animals can be moderate (e.g. poultry) to low (e.g. beef). Beef is particularly inefficient in that one calorie equivalent of beef requires approximately 10 cal equivalents of plant-derived feed. Efficiency is also dependent on the nature of the feed used. Furthermore, animal production facilities must contend with disposal of excreta and other wastes. Some researchers have noted these opportunities to improve the efficiency of animal protein production and are taking approaches that are based on GM (Phipps & Cockburn, 2003; Cockburn, 2004). Efforts are underway to improve the feed efficiency of crops (O'Quinn *et al.*, 2000) and to reduce the environmental impact of the waste produced. Assuming the demand for more animal-based protein in human diets will rise globally, the evident environmental impact of this choice should be addressed, perhaps in part using GM crops.

We have described several opportunities to improve agricultural production of food and the potential future involvement of GM in meeting the needs of the growing population. Agriculture also serves mankind by providing fuel and fibre, and millions of hectares of land are managed for this purpose. For example, demand for paper and other products derived from trees continues to increase. As demand increases, so does the pressure on the environment to produce the necessary raw materials to meet these needs. GM technology is being utilized as one solution to alleviate some of the stresses created by expansion of consumer demand in non-food products. Two examples of research underway with transgenic trees deal with reducing the lignin (Pilate *et al.*, 2002) that would have to be removed in the pulping process, and modifying trees to have accelerated growth rates (Eriksson *et al.*, 2000). Both approaches are targeting trees used in plantations, and depending on their success could result in more efficient production of wood products from fixed operations. In the longer term, transgenic trees could reduce the need for harvesting wood from natural areas such as forests, which would have clear environmental benefits globally.

2.4.5 Other GM plants

There are many other examples of GM plants and traits that are being developed that are not reviewed here. For example, plants that produce pharmaceutical and

industrial products (PMPs and PMIPs) are under development. Currently, they are also a source of great controversy concerning their risks and benefits. The focus of this chapter has been on GM crops that will impact crop production effectiveness.

2.4.6 *Gene flow containment*

The discussion above outlined the current and potential future applications of GM crops, emphasizing their positive attributes with respect to improving the effectiveness of crop production systems. However, there is much interest and concern surrounding the consequences of gene flow from GM crops. Gene flow is certainly not a new phenomenon in crop production and plant biology. According to Slatkin (1985), 'gene flow is a collective term that includes all mechanisms resulting in the movement of genes from one population to another'. Concerning plants, there are three potential mechanisms whereby gene flow could occur: movement of gametes (e.g. pollen), movement of viable plant parts such as seed and movement of segments of DNA (e.g. horizontal gene flow). The first two mechanisms are common in plants while the last one is exceedingly rare. Today, it is common for scientists and the general public to associate gene flow with terms such as outcrossing, cross-pollination, pollen flow and other convenient labels. Regardless, gene flow associated with GM crops is a complex phenomenon and discussions of the risks associated with gene flow encompass far more than the science associated with the biological facts. Current and future GM crops will have to be assessed for potential risks posed as a result of release to the environment and gene flow.

The current GM crop products have been developed and marketed largely without extraordinary measures to manage or mitigate gene flow. The risks have been determined to be acceptable or manageable based on the knowledge of the crop, the trait, the likely receiving environment and the interactions among these. Research has shown that, on a case-by-case basis, HT and virus resistance do not confer a selective advantage outside the agricultural field (Bartsch *et al.*, 1996; Snow *et al.*, 1999). Gene flow from GM Bt crops to wild relatives is of broad theoretical interest (Stewart *et al.*, 1997; ISB, 1999; Snow *et al.*, 2003), but the absence of wild relatives in areas of corn and cotton production result in a risk that is effectively zero. As such, the environmental impact associated with gene flow from the current products that have completed regulatory review has been shown to be minimal, but issues of product stewardship and volunteer management are receiving careful attention.

Nevertheless, GM affords numerous opportunities to introduce traits from novel sources and to apply new knowledge from genomics to environmental issues associated with production agriculture. New mechanisms to protect plants against biotic and abiotic stressors and novel modifications to plants that could result in nutritionally improved plants may have adverse consequences to wild and weedy compatible relatives. Gene flow from crops with these new traits will undergo rigorous scientific review. In cases where scientific analysis concludes that the impacts to compatible species are unacceptable, appropriate and effective gene flow risk mitigation will have to be evaluated prior to deregulation.

Table 2.5 Gene containment systems for GM crops

Technique	Description	Status
Maternal inheritance	Genes are maintained in mother plants by being localized in plastids using techniques like chloroplast transformation	<i>Research</i> – Transformation works in some plants but has not been shown to be 100% effective in all plants
Male sterility	Eliminate pollen production	<i>Commercial</i> – Male sterility systems have been used in hybrid seed production using GM and traditional breeding
Seed sterility	Production of seeds that do not germinate	<i>Theoretical</i> – Introduced as ‘terminator’ technology, this strategy has never been reduced to practice
Self-pollination	Alteration of pollination biology to make a flower completely self-pollinating	<i>Theoretical</i> – Plants like soybean are highly selfing, whereas maize is outcrossing. This approach would involve modifying the flower morphology of a plant
Apomixis	Fertilization without pollination	<i>Theoretical</i> – Researchers are studying the molecular basis of apomixis
Genome compatibility	Genes inserted in specific genomic locations will not introgress into compatible plants that do not share this genome	<i>Research</i> – GM crops are being selected for advancement and development based on the genomic location of the transgene
Transgenic mitigation	A technique that uses a transgene to make the offspring less fit	<i>Theoretical</i> – A complex approach predicated on the assumption that the addition of a gene will confer a significant fitness disadvantage to the progeny

Adapted from Daniell, 2002.

New technologies to mitigate gene flow are being studied in both university and industry laboratories. Approaches under investigation (Table 2.5) range from elimination of extraneous DNA to the introduction of genes that alter the offspring plants’ ability to reproduce or compete (Daniell, 2002). The basic strategies for gene containment as reviewed by Daniell (2002) include elimination of extraneous DNA via transformation technique (Zuo *et al.*, 2002), marker gene excision (Hare & Chua, 2002; Luo & Keenan, 2002; Ow, 2002; Gilbertson, 2003; Lyznik *et al.*, 2003), gene targeting to specific genomic locations (Puchta, 2002; Hanin & Paszkowski, 2003), containment in maternal plants through chloroplast transformation (Maliga, 2004) and reduction in the fitness of offspring plants (Gressel, 1999, 2002; Gressel & Al-Ahmad, 2003). It is important to note that the last technique being proposed (Gressel, 2002) is not strictly a gene flow containment system since it acts after the gene flow event in a recipient plant. It is often being described as a means to manage volunteer GM plants.

Because of the widely ranging opinions and concerns about gene flow risks and GM crops, research in this area of gene containment is attractive. However, decisions to use a gene containment system with a GM crop will require an examination of numerous complex factors that will be included in the costs and benefits analysis. First, development and validation of a gene containment system will require many years of research to prove that it works. The level of containment provided by the system will necessarily have to be matched with the gene flow risks associated with the new trait compared to other methods of risk management. As such, the environmental risks associated with the gene containment system itself will have to be assessed. If a GM approach is used, a regulatory authority will necessarily conduct a risk assessment prior to use in a commercial product. Finally, the financial costs connected to the development of both the trait of interest and the containment system will have to be recoverable. Decisions concerning gene containment systems will be complicated and have far-reaching effects on the future use of GM as a technological innovation.

2.5 Summary

This chapter attempts to provide the reader with a perspective on GM crops and their potential to improve the effectiveness of crop production systems and contribute to sustainability. Information is presented in a manner that highlights the opportunity presented by GM crops to address significant environmental issues that face the world. The growing global population is placing increased demands on agriculture to provide the food, feed and fibre requirements. In turn, farmers are responding by producing more products for more people with limited land and water resources. Furthermore, farmers are under increasing pressure to grow crops in a manner that will not compromise the needs of future generations. A basic premise of this chapter is that new technologies have helped reduce the environmental impact of farming in the past, and more tools, technological and knowledge-based, are needed to make agriculture more sustainable. Several examples of GM crops have been presented showing how traits like Bt and HT can reduce inputs such as pesticides and tillage operations, thereby reducing the use of fossil fuels in industrial systems. These products have also been shown to reduce exposure of humans and other organisms to more toxic pesticides. Future products are also likely to have similar effects in increasing agricultural efficiency by either reducing inputs or increasing outputs. Importantly, the introduction of GM crops must be done carefully recognizing that product stewardship is integral to the long-term durability of these products.

Today, crop biotechnology is in the midst of a broad, risk-centered, often emotional, debate that was virtually unknown to practitioners of traditional breeding 20 years ago. The present controversy encompasses concerns ranging from the fear that gene flow from GM crops may result in irreparable ecological damage to worries that the economic and ecological benefits of GM crops may be delayed

because of excessive regulatory burdens based on theoretical speculations with a low probability of occurrence (Hails, 2000). This polarization is evident in the opposing views on precaution, where some have cautioned about using an overly strict interpretation of the *precautionary approach* (Conko, 2003; Nuffield, 2003) for assessing GM crops. The Nuffield Council on Bioethics (2003) highlighted the impracticalities of demanding evidence of lack of risk in a discussion paper. Nuffield went further to state that there is an 'ethical obligation' to examine the potential benefits GM technology could bring to developing world agriculture.

The improvement of agriculture and food security depends on several factors. These include stable political environments, appropriate infrastructures, fair international and national agricultural policies, access to land and water, and improved crop varieties which are suited to local conditions. In focusing on current and potential uses of GM crops we therefore consider only part, albeit an important one, of a large and complex picture. However, we are clear that in particular cases, GM crops can contribute to substantial progress in improving agriculture, in parallel to the (usually slow) changes at the social-political level. GM crops have demonstrated the potential to reduce environmental degradation and to address specific health, ecological and agricultural problems which have proved less responsive to the standard tools of plant breeding and organic or conventional agricultural practices. Thus, we affirm the conclusions of our 1999 Report that there is an ethical obligation to explore these potential benefits responsibly, in order to contribute to the reduction of poverty, and to improve food security and profitable agriculture in developing countries. (Nuffield Council on Bioethics, 2003, p. xiv)

One thing that is certain is that a 'no' decision and no decision are tacit acceptance that the *status quo* is the lower risk option. In the case of agriculture and crop production, we know that *status quo* is not sustainable. Finally, the author, while respectful of the beliefs that justify the precautionary principle, is concerned about its environmental impact. Progress towards addressing meaningful environmental problems is being delayed in some parts of the world for additional information to come to light with the hope that this new knowledge will improve our ability to make decisions with more certainty. A better way forward is to pursue parallel tracks of acquiring knowledge while examining the effectiveness of new tools in production systems.

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3 Pollen dispersal vectored by wind or insects

Gavin Ramsay

3.1 Introduction

3.1.1 *The fascination with pollination*

It is no surprise that the process that generates seeds and fruits, crucial to the survival of man on this planet, has attracted interest for a very long time. Around 300 BC, Theophrastus understood that dust from the male flower of date palm and fig assisted the fruitfulness of the female tree in some way (Proctor *et al.*, 1996). Even earlier than the Greeks, a bas-relief from Assyrian culture, around 1500 BC, showed mythological creatures pollinating date palms (Real, 1983), an early indication that wind pollination in this species may have been inadequate and that its specialised insect pollinator may have been missing from some regions into which cultivated date palm was taken. However, the idea that a sexual fusion takes place in plants did not appear until the writings of several seventeenth-century investigators, which was further elaborated on by experimentation and the new science of microscopy in the following century. In 1793, Sprengel published his book *Das entdeckte Geheimniss der Natur im Bau und in der Befruchtung der Blumen*, an encyclopedia of the floral adaptations of 500 different flowers (Proctor *et al.*, 1996). Among his conclusions were that wind-pollinated flowers produce a much greater quantity of pollen than do insect-pollinated flowers, and he noted the significance of their exposed anthers and large, feathery stigmas. Charles Darwin discussed Sprengel's work with his contemporaries, and, more than 50 years after Sprengel, published his own observations on pollination biology, including his classic description of the interactions of orchids with their pollinators (Darwin, 1862).

As studies of pollination biology expanded beyond the habitats and the crops of Western Europe and North America, further complexity and diversity of pollination mechanisms and vectors were revealed. Among the pollinator–plant interactions described in the monograph by Proctor *et al.* (1996) are the fig–fig wasp symbiosis based on the provision of specially adapted sterile flowers on which the wasp depends, the insect-trapping activities of genera such as the monocot *Arum* and the asclepiad *Ceropegia* for the purposes of pollination, the frequent bat and bird pollination found in the tropics, pollination by water, by rodents, and even specific adaptations in some *Acacia* species, which facilitate pollination by browsing giraffes in the savanna of South Africa.

3.1.2 *The pollination of crop plants*

The pollination mechanisms of crop plants depend in large part on the wild species from which they are derived. The world's most important crop plants are derived from species in the Poaceae (FAO, 2004), and so are either cross-pollinated by wind or are self-pollinated. The world's most important food crop, maize, is pollinated by airborne pollen. Most of the pollen fertilising a maize plant is of non-maternal origin. Thus, although the species is self-compatible and inbred lines can be produced, in reality the species is an outbreeder, requiring airborne pollen for normal yield. Among the world's other major cereal crops, sorghum, millet and rye are also mostly outcrossed and depend on airborne pollen. Outcrossing is encouraged in these species by the large amount of pollen produced by anthers that become exerted at anthesis (e.g. rye, maize), by the asynchronous maturity of male and female floral parts, and in maize by the separation of male and female flowers on different structures. In crops such as rice, wheat, barley and oats, most pollination is by self-pollen, encouraged by florets that gape less widely. Even in these species, however, a low percentage of pollination takes place with pollen from other plants. Grass as forage, often sown as elite cultivars and mixtures in high-yielding temperate forage production, is also a crucial element of the food supply. Most of the forage grasses are wind-pollinated, outcrossing species (Figure 3.1). Other crops of local importance in certain regions, pollinated primarily by pollen borne on the wind, include oil palm and beet. Considering only those crop species with at least 10 000 Mt of production in 2003 (presented in Table 3.1) and ignoring forage plants, around 40% of world production comes from crops mostly pollinated by the wind.

Although the species discussed above are essentially anemophilous species, their flowers also attract insects to consume or collect pollen, which in the process may effect pollination. For example, several species of hoverfly consume the pollen of wind-pollinated plants such as grasses and *Plantago*, and, in the process of doing so, are thought to cause incidental cross-pollination (Gilbert, 1981). In some cases,

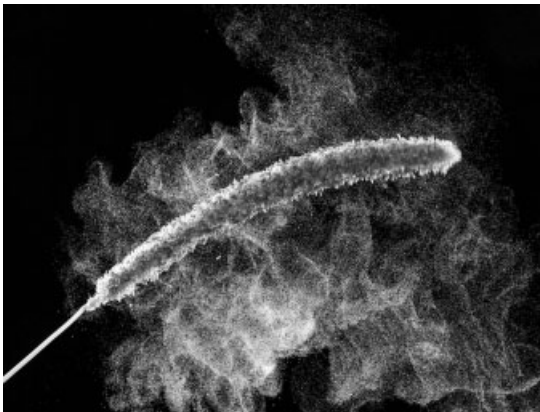


Figure 3.1 Pollen shedding in timothy grass (*Phleum pratense* L.). Courtesy of Stewart Malecki, SCRI.

Table 3.1 World crops: their pollination requirements and production in 2003

Crop ¹	Production (Mt × 1000)	Harvested product	Pollination ²
Cereals			
Maize	638.0	Seed	Wind
Rice, paddy	589.1	Seed	Self (wind)
Wheat	556.3	Seed	Self (wind)
Barley	141.5	Seed	Self (wind)
Sorghum	59.6	Seed	Wind
Millet	29.8	Seed	Wind
Oats	26.3	Seed	Self (wind)
Rye	14.9	Seed	Wind
Triticale	10.2	Seed	Self (wind)
Roots, tubers and vegetables			
Potatoes	310.8	Root/tuber	N/a ³
Cassava	189.1	Root/tuber	N/a
Sweet potatoes	121.9	Root/tuber	N/a
Cabbages	66.0	Vegetative	Insect ⁴
Onions, dry	52.5	Root/tuber	Insect
Yams	39.9	Root/tuber	N/a
Carrots	23.3	Root/tuber	Insect
Lettuce	20.8	Vegetative	Self (insect)
Cauliflower	15.9	Vegetative	Insect
Garlic	13.7	Root/tuber	N/a
Spinach	11.9	Vegetative	Wind (insect)
Fruits			
Tomatoes	113.3	Fruit	Self (insect)
Watermelons	91.8	Fruit	Insect
Bananas	69.3	Fruit	Parthenocarpic ⁵
Grapes	60.9	Fruit	Insect
Oranges	60.0	Fruit	Insect
Apples	58.0	Fruit	Insect
Cucumbers and gherkins	39.6	Fruit	Insect
Plantains	33.0	Fruit	Insect
Eggplants	29.0	Fruit	Insect
Melons	26.7	Fruit	Insect
Mangoes	25.6	Fruit	Insect
Capsicum	23.2	Fruit	Insect
Other citrus	21.0	Fruit	Insect
Pumpkins, squash and gourds	19.0	Fruit	Insect
Pears	17.2	Fruit	Insect
Peaches and nectarines	14.8	Fruit	Insect
Pineapples	14.6	Fruit	Parthenocarpic
Lemons and limes	12.5	Fruit	Insect
Plums	10.1	Fruit	Insect
Grain legumes			
Beans, dry	19.0	Seed	Self (insect)
Peas, dry	10.2	Seed	Self

(Continued)

Table 3.1 World crops: their pollination requirements and production in 2003 (*Continued*)

Crop ¹	Production (Mt × 1000)	Harvested product	Pollination ²
Oil and industrial crops			
Sugarcane	1333.3	Vegetative	Wind
Sugar beet	233.5	Vegetative	Wind (insect)
Soybean	189.2	Seed	Self
Oil palm fruit	143.4	Fruit	Insect and wind
Seed cotton	56.1	Fruit	Insect
Coconut	52.9	Seed	Insect
Rapeseed	36.1	Seed	Self and insect
Groundnut	35.7	Seed	Self
Sunflower	27.7	Seed	Insect
Olive	17.2	Fruit	Wind and self (insect)

¹ Of the 144 commodities monitored by FAO, only those with production of at least 10 000 Mt are presented here.

² Where a vegetative part of the plant is harvested, this refers to pollination during seed production.

³ N/a — not applicable, propagated and harvested vegetatively, although potatoes are grown from true seed in some parts of the world.

⁴ This and other fruits may have parthenocarpic forms.

⁵ Wild and seeded forms are pollinated by hummingbirds.

certain insects have evolved associations with particular species that may otherwise be wind-pollinated species. The oil palm has an association with the weevil, and this has led to the weevil being deliberately introduced to Malaysia for the purpose of pollinating plantation palms (Syed *et al.*, 1982). Honeybees are known occasionally to collect the pollen of grass species, and our own observations indicate that *Bombus* species will do this too. Particularly in environments where other pollen sources may be scarce, bees will gather significant quantities of pollen from maize plants (Nowakowski & Morse, 1982). However, in the case of maize plants, particularly with the separation of male and female flowers on the stem and the lack of a reward from the female flowers, the release of pollen from one field of maize over the stigmas of plants in another by honeybees seems likely to be a rare event.

Although the world's most important food crops are cereals, clearly adapted for wind-mediated pollination, a very large number of other crops require insects for efficient pollination and seed or fruit production. McGregor (1976) estimates that about one third of all food consumed is attributable, directly or indirectly, to insect pollination. In Table 3.1, most of the crops listed in the top 51 require, or benefit from, insect pollination, at least during the seed production phase. In most cases the primary insect pollinators are bees. As agricultural production has intensified, populations of native pollinators have declined, because of both habitat loss and the use of pesticides. Bees have been noted to be pollinators for 77% of 82 species commodities, and are the most important known pollinator for 48% of them (Prescott-Allen & Prescott-Allen, 1990; Delaplane & Mayer, 2000). Various figures have been derived for the value to the economy of this pollination effort. In the United States, the value of honeybee pollination was placed at \$9 billion in 1989 (Robinson *et al.*, 1989). For the European Union (EU), the value of honeybee pollination was

placed at €4.3 billion in 1989 (Delaplane & Mayer 2000). Such a high dependence on managed and wild bee and other insect pollinators for sustainable cropping requires more than managing crop agronomy effectively. An integrated approach to the whole system is required, ensuring that introduced, managed pollinators are not damaged by agricultural practices, and that wild pollinators together with the surrounding habitats that support them are maintained for current and future generations.

3.1.3 *Pollen dispersal, gene flow and GM crops*

Genetic impurity can be introduced into harvested products by various means. Unwanted genotypes, whether GM or not, can appear in a seed stock owing to physical mixing after harvest, seedbank persistence, transfer on farm machinery and other means. Cross-pollination excites most attention perhaps because of the uncontrolled and involuntary nature of the process, and this source of impurity has received much publicity in scientific, public and political forums in recent years. As research has revealed new surprises in terms of distance or level of cross-pollination, media interest has often been intense. The public, regulators, politicians and media turned to science to deliver answers on the likely impact on purity of cross-pollination in different circumstances. Numerous studies conducted on crops that are now available in GM form provide a body of evidence on the effect of separation distances on cross-pollination rates and the implication for purity thresholds, based on single-field comparisons (e.g. Ingram, 2000). However, initial trials on small plots were generally insufficient to predict the processes operating as trials were scaled up to full-scale release. Those experiments that gave an early indication that field-sized sources of pollen behave in ways unlike small plots (Timmons *et al.*, 1995) generated controversy and much media interest, because they appeared to challenge the validity of the separation distances used at the time.

Some crops can be pollinated by a number of different vectors. This uncertainty, together with the general lack of convincing experiments that are able to partition the effects of different vectors, has led to confusion over the processes and routes of pollen transfer in some species. The aims of this chapter are to put the pollination of crops into the context of the breeding systems developed by their wild ancestors, to attempt to clarify the importance of different vectors to different crop species and to show the way towards better, more generic means of considering pollen movement and hence improve the understanding of gene flow in all crops.

3.2 **Evolutionary and ecological aspects of pollination biology**

3.2.1 *Evolutionary aspects of wind-mediated pollination*

Wind as a means of dispersing sexual propagules in plants occurred as early as the beginning of the emergence of plants onto the land 420 million years ago (Habgood *et al.*, 2002). However, the spores of these primitive plants, together with those of bryophytes and pteridophytes, are not analogous to the pollen grains

of higher plants and gymnosperms. The evolution of flowering plants was associated with a concurrent reduction in the gametophyte generation so that it became represented by few-celled structures, namely the pollen grain (microgametophyte) and embryo sac (megagametophyte). The equivalent of the spore itself, the pollen or embryo sac mother cell, remains within the floral structures of the higher plant. It seems likely that the early angiosperms bore a greater resemblance to the families Magnoliaceae and Ranunculaceae than other extant families (Sporne, 1971). As both early dicotyledonous and monocotyledonous plants appear to have been petaloid, it is likely that they were pollinated by insects and so that anemophily is probably a derived trait in both groups. The grasses and sedges (Poaceae and Cyperaceae) are two entirely anemophilous monocotyledonous families. Among the dicotyledonous angiosperms, the most conspicuous anemophilous plants comprise a diverse set of catkin-bearing temperate tree species. Clearly, anemophily has sufficient advantages to drive the evolution of these anemophilous groups in the first place, and to place these groups in such a dominant position in some of the world's major habitats. Mankind should be grateful for this advantage gained by the grasses in particular, as this family has provided the crop species that coevolved with early man and permitted his move to an agrarian lifestyle, and of course the ultimate development of civilisation.

It is curious that there is a temperate–tropical dichotomy for tree pollination type. Temperate forest trees are dominated by early-season anemophily and, within their habitat, are often dominant or at least patch-forming species. These species come from a relatively restricted group of plants including the gymnosperms *Abies*, *Larix*, *Juniperus*, *Picea*, *Pinus*, *Taxus* and others, the three related angiosperm families Betulaceae (*Betula* and *Alnus*), Corylaceae (*Carpinus* and *Corylus*) and Fagaceae (*Castanea*, *Fagus*, *Nothofagus* and *Quercus*) and scattered genera in other families such as Oleaceae (*Fraxinus*), Saliceae (*Populus*) and Ulmaceae (*Ulmus* and *Celtis*). Few major temperate forest trees exhibit entomophily and those that do are less frequently the dominant types in their habitat (*Acer*, *Tilia*, *Salix* and *Prunus*, for example). In contrast, tropical forest is populated by a great diversity of scattered individuals of entomophilous and other animal-pollinated tree species. The patch-forming nature of anemophilous species extends to many terrestrial and herbaceous as well as arboreal species. As with temperate forest trees, these come from a restricted set of families including, in western Europe, the Poaceae, Cyperaceae, Urticaceae, Polygonaceae (*Rumex*) and Euphorbiaceae (*Mercurialis*). The prevalence of patch-forming anemophilous species among herbaceous plants adds to the view that to succeed with a wind-mediated pollination system, locally high densities of plants can be an advantage. This property has resonance for species grown as crops in high densities within agricultural fields.

Other habitats with low numbers of insects, such as salt marshes and some semi-arid habitats, also have high levels of anemophily (Cox, 1991). The interactions in these cases may be even more complex, with the possibility that, as water use efficiency of the plant increases, nectar secretion becomes a greater cost for the plant, and both the attractiveness of the individual plant and the sustaining capacity of the

Table 3.2 Adaptations, traits and environmental variables that tend to be associated with entomophily and anemophily

Trait	Entomophily	Anemophily
<i>Environmental variables, particularly relating to tree species</i>		
Air movement	Relatively low air movement	Seasonally windy
Humidity	Often high	Variable
Community structure	Scattered individuals	Patches or dominant
<i>Floral traits</i>		
Pollen morphology	Often papillate, highly sculptured or with threads, aggregating	Often with little surface detail, friable
Pollen size	Variable	Often small
Stigma type	Often capitate, discoid or compact	Usually elongated and linear, often branched
Flower presentation	Variable exposure, often rigid	Always exposed, male flowers often loose or articulated
Flower morphology	Usually petaloid	Almost never petaloid
Phenology	Commonly avoiding cold or dry seasons	In trees, commonly before foliar bud break
Scent	Often scent producing	Never scented
Other reward	May produce nectar or wax bodies	No rewards offered
<i>Fruit</i>		
Fruit type	Various, often fleshy in trees	Often few and sometimes large-seeded, seldom fleshy in trees

habitat for a diversity of insect pollinators reduce. These pressures would tend to favour anemophilous plants, or plants that restrict their flowering to brief periods of adequate water supply. Some of these features of the habitats favouring anemophily are summarised in Table 3.2. It is also striking that in desert habitats, after heavy rains, the entomophilous element of the flora can be conspicuous and the insect activity intense during this short window (Proctor *et al.*, 1996), indicating even within habitats, partitioning of preferred pollination biology according to season may take place.

3.2.2 Adaptations for wind pollination

Anemophily brings certain costs. There is a suite of traits associated with anemophily that are reduced or absent in entomophilous plants. Pollen is often produced on massed structures such as catkins or panicles, helping to generate very large numbers of pollen grains. The anthers themselves tend to be borne on structures that expose them to drying and mobilising wind currents (loose catkins, or long thin filaments lifting them away from the structure), whereas ovaries are retained in more enclosed structures. Styles are often delicate, exerted and short-lived, adapted for maximal sampling of passing air currents. In many cases, stigmas have become elongated

into long linear filaments, the most efficient structure for the impaction of pollen borne in airstreams providing both a large surface area and a minimal resistance to airflow through the structure.

Aerodynamic impaction on surfaces presented to the airstream is a major means of collecting pollen suspended in the air. Consequently, anemophilous plants have evolved to reduce the risk of pollen being filtered out of the airstream. Temperate deciduous trees generally flower in advance of leaf bud break, at a time when the forest canopy is at its most porous. Furthermore, neither male nor female flowers retain petals or conspicuous bracts, also serving to reduce the filtering effect on airborne pollen. Herbaceous anemophilous plants flower at a variety of times. They reduce the unwanted impaction on foliage and maximise their exposure to air currents in most cases by lifting their male flowers above the foliage.

Enormous quantities of pollen can be required to achieve successful wind pollination, depending on the size of the plant and its tolerance for inbreeding. For example, in *Quercus*, a dominant deciduous forest tree genus in large areas of temperate forest on both sides of the Atlantic, if one pollen grain needs to settle on the square millimetre of the stigma, every square metre of the plant's habitat needs to receive around 1 million pollen grains for successful seed set. Pollen production of *Quercus* is said to be sufficient for this amount of pollen rain, and the total annual pollen rain may be around 300 million grains $\text{m}^{-2} \text{y}^{-1}$ in the heavily forested landscape of Sweden (Erdtman, 1969).

Anemophilous pollen is normally less ornate than the sticky types of pollen adapted for transport on insects. It seems likely that electrostatic charge may have a role in the launching of pollen into the air, its maintenance in airstreams, and its attraction to plant surfaces. Many anemophilous species have smaller pollen than those of entomophilous plants. Both settling through the air and impacting on surfaces from a moving airstream may remove pollen from the air. Sedimentation rate, or the terminal velocity of a falling pollen grain, depends on its size, varying from 2.2 cm s^{-1} for *Salix caprea* to 39 cm s^{-1} for *Abies alba* (Proctor *et al.*, 1996). The processes that act to keep pollen in the airstream will be discussed in more detail later in the chapter.

3.2.3 Airborne pollen recording for allergy sufferers

As allergies become more prevalent in Western society, more people are keen to receive warning of high-risk days for allergic rhinitis, otherwise known as hay fever. In the United Kingdom and over much of western Europe, the major allergenic pollen types are the many species of the Poaceae in the summer, and the several species of trees that flower in the early spring. National networks in the United Kingdom and elsewhere monitor airborne pollen daily through the summer using volumetric spore traps such as Burkard traps (Figure 3.2). Large data banks of aerial pollen flora have been created at several sites. The daily counts, local weather forecasts and experience of trends in previous seasons together permit accurate forecasts for the days ahead, which are propagated through various media outlets for the benefit of



Figure 3.2 A Burkard volumetric spore trap in use for pollen monitoring.

allergy sufferers. These data sets include all identifiable pollen types throughout the season. The pollen recovered in these traps is often dominated by local sources, but a component comes from substantial distances. At the SCRI (Scottish Crop Research Institute) site in eastern Scotland for example, the abundant conifer pollen and seasonally frequent *Brassica* pollen will come from forests and fields at long distances at least tens of kilometres away in the case of conifer pollen. It is likely that a significant proportion of the pollen recorded comes from even further away, and the large clouds of *Betula* pollen recorded across western Europe in some years may be an indication of the scale of pollen transport (Figure 3.3). Tree pollen has been recorded at high concentrations in conditions of strong convection at 2000 m altitude above Göttingen, implying the transport of pollen over hundreds of kilometres per day.

The methods employed by these monitoring sites measure the number of pollen grains suspended in the air rather than deposited on static traps. Around 10 grains of an allergic type of pollen per cubic metre is considered to be a sufficient trigger for rhinitis in susceptible individuals, and daily averages in the United Kingdom can rise as high as 300 m^{-3} or so for tree species such as birch, and 200 m^{-3} or so for grass pollen during the peaks in midsummer.

3.2.4 Evolutionary and ecological aspects of entomophily

In temperate forests, entomophily is common in plants of the shrub layer and the ground flora. Its infrequent occurrence in the dominant tree flora of northern forests is striking, perhaps because of a combination of the lack of sufficient populations

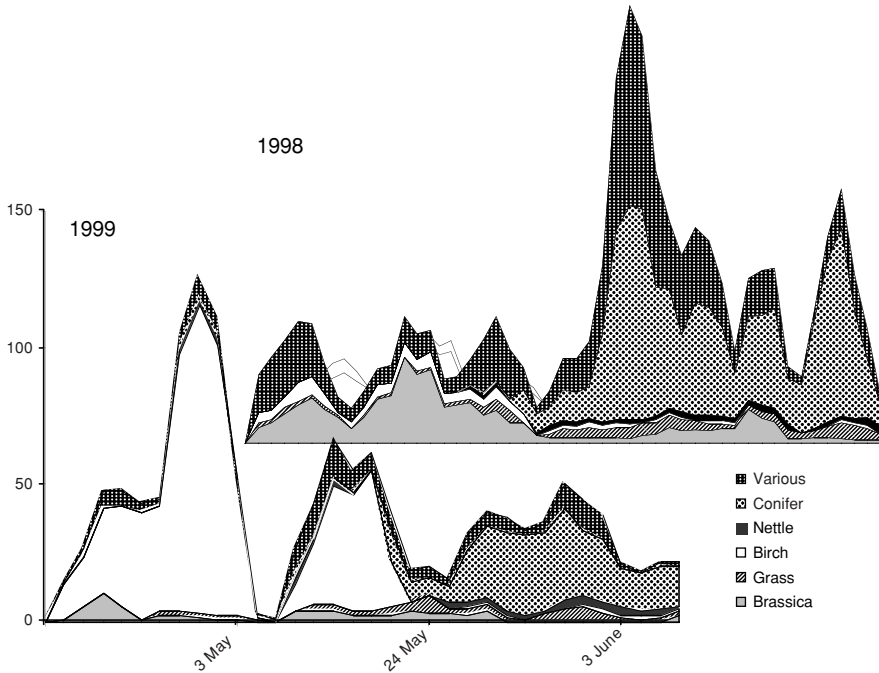


Figure 3.3 Airborne pollen (grains m^{-3} , data smoothed across days) through springtime in two years in eastern Scotland, with birch and brassica pollen showing contrasting levels.

of pollinators early in the season, the lack of a need to encourage precise delivery of pollen across large distances and to some extent the constraints of the phylogeny of the species of tree involved. In plants below the canopy of these forests, entomophily is much more common. Although cause and effect are hard to disentangle, entomophily may be encouraged or permitted by less massed flowering with a concomitant lower atmospheric pollen load and a greater need for the selective positioning of pollen on distant congeners. The more sheltered habitat also provides a more congenial environment for pollinating insects.

In contrast to temperate forests, tropical forest trees are mostly pollinated by insects, particularly bees (Bawa, 1990). Other pollinators of tropical forest trees include bats and hummingbirds, but anemophily is unusual (Proctor *et al.*, 1996; Dick *et al.*, 2003). The factors encouraging these modes of pollination may include the need for precise pollen delivery to widely scattered individuals (of course the corollary is also true, that entomophily permits scattered population to exchange genes), local weather including high humidity and regular precipitation and the phylogenetic background of the species encountered in this habitat.

There appears to be an association between pollen and seed dispersal mechanisms. Trees in climatic zones with high levels of anemophily appear to have an association with winged seeds for wind dispersal, or nuts. Proctor *et al.* (1996) note that large seeds are often associated with climax forest trees that require seedlings to have sufficient reserves to permit establishment in the shade of the forest floor. The production of fewer but larger seeds per flower requires few pollen grains on each stigma, and this appears to be a trait exhibited by many wind-pollinated species. The complex traits, such as few seeds per ovary, large seeds with autumn dispersal and a long development period and early flowering to avoid the pollen-collecting nature of dense foliage, all appear to reinforce anemophily. In contrast, frugivory as a seed dispersal strategy appears at higher frequency in species and habitats in which insect-mediated pollination predominates. In this case, the evolutionary forces at work are less clear, although it is possible that the need for more efficient dispersal away from the mother plant is one of these forces.

3.2.5 Adaptations for entomophily

In contrast to adaptations for anemophily, entomophily encourages the development of showy corollas or bracts, and the limited production of sticky and often ornate pollen grains, sometimes with adhesion threads. Flowers may also produce scent, secrete nectar, have additional visual cues such as nectar guides or produce other floral rewards such as wax bodies. Given the intense coevolution between pollinating animals and the angiosperms throughout the existence of the latter, it is not surprising that there is an enormous number of diverse adaptations throughout the flowering plants to encourage the successful movement of pollen by insects. There are specialised mechanisms to glue pollen onto insects and flower morphologies to protect pollen from the elements, to mete out quantities of pollen, to package pollen for the pollinator and to present it appropriately. Specialised adaptation of flowers reaches a peak of complexity in the orchids as was described by Darwin (1862). Some general attributes of entomophily are given in Table 3.2. Among the major crops grown in temperate zones, oilseed rape (*Brassica napus*) demonstrates many of the features necessary for the successful partnership of a flowering plant with generalist pollinators (Figure 3.4). Conspicuous petals serve to attract from a long distance, and in massed populations such as fields they are visible from distances of around 10 km. Odour is also used to recruit and guide pollinators, and may be an even more potent cue than the sight for insects commuting long distances to reach populations of the species. *Brassica* flowers are open and offer no resistance to any of the major pollinators. Even long-corolla specialists such as *Bombus hortorum* visit oilseed rape flowers, presumably attracted by the seasonally abundant nectar resource (Gordon *et al.*, 2002). Most oilseed rape flowers offer a plentiful supply of pollen for collection, and the abundant secretion of a high sugar nectar permits this crop to be the major source of nectar for honey production in the United Kingdom (Carreck *et al.*, 1997).



Figure 3.4 Oilseed rape flower showing adaptations for entomophily: showy petals, small stigma presented to incoming insects, stamens at two levels, and a dark nectary is visible at the base of a petal. Leaving a stamen is a pollen beetle, *Meligethes aeneus*, a pest species that also effects pollination.

3.3 Managing insect pollination for crop production

3.3.1 Crops benefiting from wild and managed pollinators

A wide range of crop plants benefit from insect pollination (Table 3.1). In the review by Delaplane and Mayer (2000), 36 different crops grown in the United States are described in detail, together with their requirements for pollinating insects, recommended stocking rates for introduced honeybee colonies and a review of the literature on the effect of pollinators for each crop. Top fruit (*Pyrus*, *Malus* and *Prunus*) and soft fruit (*Rubus*, *Fragaria*, *Vaccinium* and *Actinidia*) are very dependent on pollination, either for fruit set or for enhancing the growth of the fruits that do set. In eastern Scotland, Willmer *et al.* (1994) investigated the relative efficiency of the main pollinators for raspberry, *Rubus idaeus* L. Individuals of *Bombus* species were more numerous, carried more pollen, worked the flowers for a greater part of the day and made longer journeys between plants than honeybees, the other abundant pollinator working the flowers. Other crop groups benefiting from, or requiring, the services of insects for pollination include brassicas, cucurbits, sunflower, various vegetable and glasshouse crops, cotton, tomato, some grain and many forage legumes. In open ground, mixed habitats these crops may be successfully pollinated by native wild pollinators. Some are capable of a degree of self-pollination. However, the introduction of managed pollinators is often necessary for full pollination and high yields of seeds and fruits in extensive monoculture in habitats where pollinators are depleted and particularly in protected cropping systems.

3.3.2 The use of managed pollinators

The advantages of introducing managed pollinators for crop pollination depend on the availability and suitability of native populations of pollinators and the reliability of their appearance in the fields. For many insect-pollinated crops in many situations,



Figure 3.5 Honeybee colonies moved near oilseed rape for honey production at Inchtute, Perthshire, Scotland.

the introduction of managed pollinators and payment for the service is economically worthwhile for the grower involved. Honeybee colonies require to be moved, need to be managed to maximise their effectiveness at the right season, and often suffer from reduced honey production on the crops and at the densities used for pollination services. In the United Kingdom, about 15% of the colonies of medium- and large-scale beekeepers were moved specifically for pollination services, mostly for top fruit (Williams *et al.*, 1993). Many more colonies are moved primarily for honey crops to oilseed rape, field beans and raspberries, where the beekeeper receives no payment (Figure 3.5). For these crops, the economic gain by the grower because of introduced pollinators is less clear, and the beekeeper is willing to move bees near these crops as they provide the nectar for his honey harvest. In Canada and the United States, there are many beekeepers providing pollination services. Each beekeeper may maintain up to several thousand hives specifically for pollination. Every colony moved under contract to a crop will earn a fee which, in the United States in 1996, earned the beekeeper an average of \$31 per hive per contract (Delaplane & Mayer, 2000).

In addition to the use of managed honeybees, in recent years there has been a large expansion of the use of several other bee species, partly driven by the greatly increased protected cropping in glasshouses and polythene houses and the relative unsuitability of honeybees for these situations. Concerns over the introduction of non-native pollinators have also stimulated the development of additional species for pollination. Several *Bombus* species are now raised in artificial nests with compartments for brood raising and the supply of sugar syrup. These are used extensively for glasshouse vegetable and fruit production, and for fruit production in polythene

houses. The advantages of bumblebees over honeybees include their foraging over more hours in the day, their ability to work some closed flowers, particularly the larger legumes that are rarely worked by honeybees, their greater acceptance of confinement in glasshouses and their ability to perform buzz pollination, pollen gathering by vibrating flowers, particularly those of tomato and eggplant (Delaplane & Mayer, 2000). Several species of solitary bee are also managed for pollination. Alkali bees (*Nomia melandria*) are successfully managed, particularly for lucerne and onion production, by creating artificial nest sites in bare ground, with selected introductions where necessary. Leaf-cutter bees from the genus *Megachile*, particularly *M. rotundata*, are raised in the spaces between corrugated sheets of different materials, and located in structures in the field giving the nests shelter. By 1988, these leaf-cutter bee systems enabled western Canada to turn from an importer of lucerne seed to an annual exporter of 1.1 million kilograms of seed (Delaplane & Mayer, 2000). Mason bees (*Osmia* species) are another group of solitary but community-nesting bees that are managed for pollination in some parts of the world, particularly Japan but now also the United Kingdom. From the viewpoint of GM risk assessment, the widespread use and movement of managed pollinators affects, and may complicate, the prospect of assembling models to predict gene flow from such insect-pollinated crops. However, where pollination is primarily achieved by introduced pollinators, the task of predicting gene flow on the landscape scale ultimately becomes simpler, as the behaviour of these pollinators becomes better known. As will be discussed later, there is also the possibility that the use of introduced pollinators can be managed to reduce pollen dispersal.

3.4 Experiments and observations on vectors in oilseed rape, beet and maize

3.4.1 Uncertainties on the relative importance of different vectors in oilseed rape

The structure of the *Brassica* flower is well adapted to generalist insect pollinators including the honeybee (Free, 1970). Compact stigmas, petals, scent production and the continuous production of nectar during the active life of a flower all contribute (see Figure 3.4 for morphological features) to its appeal. Even though *B. napus* oilseed rape is self-fertile, some authors (e.g. Eisikowitch, 1981) report that insect pollination is required for full seed production in this crop. However, some authors have encouraged the idea that oilseed rape may be wind-pollinated. The differing objectives of various studies may explain some of these differences in interpretation. For example, Williams (1984) was addressing the question of the cause of the seed set inside agricultural fields in a study of the concentrations of airborne pollen over oilseed rape fields. In this context, with intimate contact between flowers of this self-fertile crop, the touching and rubbing of inflorescences, with the massive quantities of airborne pollen within the canopy, insects may well be unimportant.

McCartney and Lacey (1991) performed an extensive study of the quantities of pollen found over and around oilseed rape crops. The highest concentrations were found on days of high radiation and wind, and lowest on days with rain. More than 60% of the pollen emitted from the field was still airborne 100 m downwind, but at ground level the concentration was between 2 and 10% of that at the edge of the crop. The authors were, however, careful to record that the relative proportions of wind- and insect-mediated pollination in oilseed rape were still unknown.

Mesquida and Renard (1982), using large cages over plots of male sterile oilseed rape, observed some seed set proximal to the pollen source, but low levels of seed set elsewhere. This appears to suggest that extreme proximity can permit high levels of seed set, a finding that is not incompatible with the observations of Williams (1984). In a later study, Mesquida *et al.* (1988) investigated the effect of excluding insects on a range of yield components in fully fertile oilseed rape. Although overall yield was unaffected, the presence of bees brought about larger numbers of seeds per pod, less secondary branching and a generally earlier and more uniform seed set. These authors listed 17 studies on oilseed rape that attempted to determine whether bees had a beneficial effect on yield: 8 recorded insignificant effects, whereas the remainder suggested that the presence of bees gives increases in yield or at least gives an earlier and more consistent seed set. With the exception of the study of Mesquida and Renard (1982), there are very few studies that address the question of the relative importance of wind and insects as pollen vectors for the successful pollination of plants beyond the confines of the source field.

3.4.2 *Oilseed rape cross-pollination: observations*

Some authors have implicitly acknowledged that insects and, in particular, bees may be important for the pollination of oilseed rape across plots and fields. In their design consisting of a 9 m circle surrounded by a 1 ha plot, Dale and Scheffler (1996) found that cross-pollination fell to undetectable levels by 24 m. Subsequent work by the same team (Scheffler *et al.*, 1995) came to the conclusion that 'the data indicated that most of the pollen was transported by bees'. The reasons given for this view were lack of association with wind direction during the experiment, the relative abundance of bees at the site (around 1 bee m⁻² flowering crop) and the confirmation that the insects were foraging on rape flowers.

The paper by Simpson *et al.* (1999) looked at wind direction in their experiment and stated that the wind direction during the experiment correlated with maximal cross-pollination. However, these authors have acknowledged (personal communication, 1999) that they misinterpreted the weather records and that there was in fact no correlation between wind direction and gene flow. Ramsay *et al.* (2003) also investigated the influence of wind on the pollination events that carried non-GM herbicide tolerance in pollen several kilometres away from the source in Tayside, Scotland. In this case a mini-meteorological station placed at the source field recorded wind flows throughout the experiment and it was apparent that the herbicide-tolerant trait was appearing as frequently in plants 4 km upwind as in other directions. Additional



Figure 3.6 Honeybee forager returning with pollen loads having visited oilseed rape.

observations suggested that wind-borne pollen was unlikely to be the cause of fertilisation observed on male sterile plants. Thompson *et al.* (1999) demonstrated that even at distant sites where overall levels of pollination were low and airborne pollen was sparse and mostly present as single grains, single pollination events often produced many seeds per fruit. This suggested that insect visits were most likely to be giving these fertilisation events. Some of the insects noted visiting flowers in the experimental area are shown in Figures 3.6 and 3.7.

Cresswell *et al.* (2003) suggest that the floral architecture of *Brassica* racemes does not lend itself to cross-pollination by wind. Any appreciable wind flow turns the



Figure 3.7 Insect pollinators on oilseed rape: (a) bee-fly (*Bombylius major*), a bumblebee mimic, (b) hoverfly (Syrphidae), (c) cabbage seed weevil (*Ceutorhynchus assimilis*) and (d) carder bee (*Bombus pascuorum*).

flower away from the direction of the wind current, sheltering the stigma. The large attenuation in pollen concentrations with distance from the crop makes appreciable cross-pollination via wind-borne pollen unlikely.

3.4.3 *Oilseed rape cross pollination: experimental data*

A limited number of experiments have been conducted that look specifically at partitioning the wind and insect elements of cross-pollination outside fields in oilseed rape. Timmons *et al.* (1995) observed seed set on de-petalled flowers, intending to discourage insect visits. Seed set was obtained in 2 years at 1.5 and 2.5 km from the nearest source at a site that received airborne pollen on volumetric spore traps, and this was taken as suggestive evidence that wind was the most important vector for oilseed rape. Hayter and Cresswell (2003) also de-petalled flowers and found that pollen accumulation on the stigma reduced about 4-fold to 10-fold, depending on the bee density in the field, but was not eliminated.

Ramsay *et al.* (2003) reported on an experiment that placed replicated batches of male sterile plants at a variety of distances from a source, and covered one batch of plants at each site with a cage of coarse netting. Although such experiments are not completely conclusive because airborne pollen kinetics may be disturbed, and because small insects were able to cross the mesh of the cage, it was clear that the netting abolished most of the cross-pollination taking place in the open. Furthermore, estimates of pollen deposition were made inside and outside cages, and no difference in oilseed rape pollen deposition could be detected (Figure 3.8). The difference between the two treatments, expressed as numbers of seeds produced, was about 10-fold. Mesquida and Renard (1982) also used cages to investigate the effect of the exclusion of pollinating insects on seed set in male sterile oilseed rape. In this case the cages reduced airborne pollen to approximately half over all tests. They found that seed production fell to between 4 and 16% of controls at 24 m and to 1 to 4% of controls at greater distances. To date, these two studies appear to give the only quantitative estimates of the difference between airborne and insect-mediated elements of cross-pollination in oilseed rape. Such studies have importance for the risk assessment process in helping to parameterise models that aim to predict pollen-mediated gene flow from GM crops to non-GM fields and wild relatives. Difficulty remains in assembling robust, predictive models that can combine the minor but easily modelled wind dispersal component with elements that describe the pattern of pollen movement mediated by several insect vectors on a landscape scale.

3.4.4 *Other crops*

There are no experiments described that permit the partitioning of pollination between insects and wind for beet crops. Beet is widely acknowledged to be primarily wind-pollinated, although there is a component of pollination contributed by insects (Dark 1971; Free *et al.* 1975). Saeglitz *et al.* (2000) also considered that insect- and

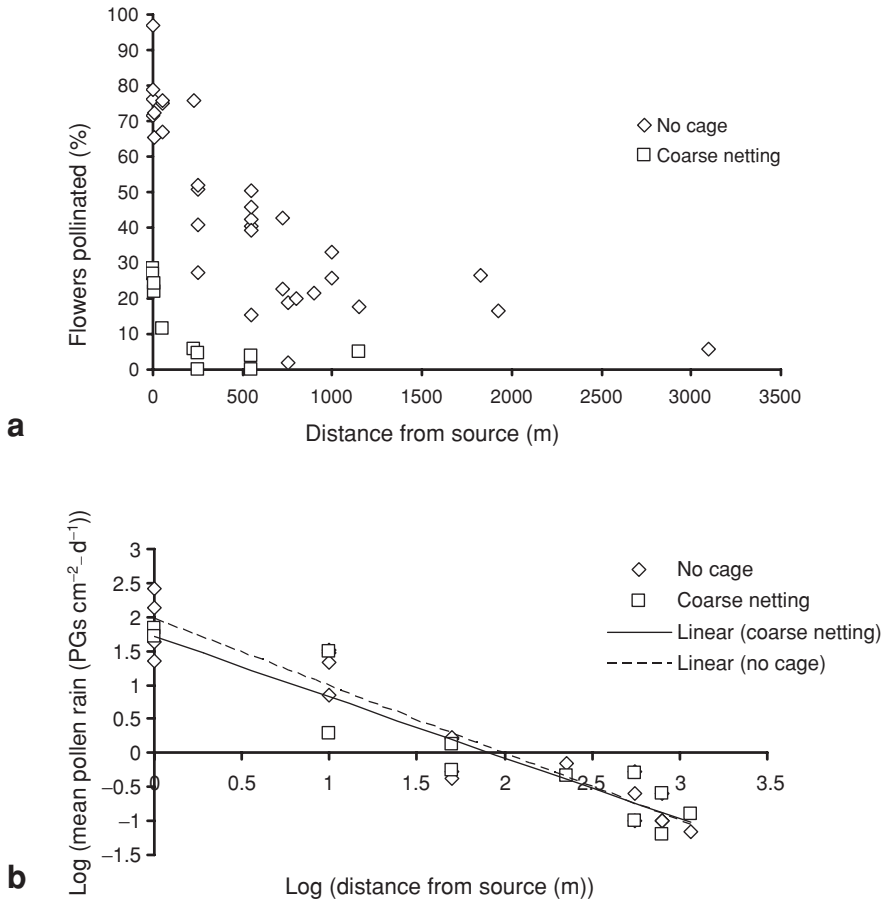


Figure 3.8 Experiments with cages: the effect of screening male sterile oilseed rape flowers from larger insect pollinators. (a) Pollination (percentage of flowers) against distance from source in the open and under cages; (b) airborne pollen determined by impaction on slides in the open and under cages, indicating that the cage had little effect on airborne pollen.

bee-mediated pollen transfer was possible in their experiments, and bees were noted visiting beet flowers. As a wide variety of insects, including bees, flies and coleopterans, have been seen on beet flowers, it can be expected that the characteristics of pollen dispersal by these insects will reflect the patterns of pollination in beet to a small extent, and that patterns of dispersal seen with airborne pollen will predominate in this crop. The origin of the few very long-distance pollination events described for beet must await experimental investigation.

In maize, the fact that bees and other insects will work the crop for pollen has been mentioned previously. However, the author is not aware of any experimental work that attempted to partition maize pollination to the various vectors.

3.5 Processes and patterns with wind-mediated pollination

3.5.1 Deposition, turbulence and impaction

Pollen is often lifted by air currents from the anthers where it was produced, and the frequently articulated or unstable structures on which they grow appear to aid the process by exaggerating movements of the floral structure when disturbed by wind. Air speed appears to be one of the main weather variables determining whether large quantities of pollen become airborne (McCartney & Lacey, 1991), and this factor may contribute to the lack of success of anemophily in some tropical habitats, as already discussed. Once airborne, pollen tends to settle at a rate that depends in part on the size of the pollen grain. A number of forces act to counteract this settling, maintaining pollen in the air for periods up to several hours (Figure 3.9). There is normally intense gravitational deposition of pollen near to the source, perhaps accentuated by clumping and other factors. Turbulence is the main cause of the continued suspension of pollen in the air. Pollen will, of course, move with the prevailing wind, but eddies in moving air cause continual mixing of the airborne pollen mass and repeatedly counteract any tendency to settle. Nevertheless, impaction onto stationary objects filters out many pollen grains. Particles in airstreams passing over objects are likely to impact on them, and here the mass of the particle has an important effect, since large particles are more likely to impact than smaller ones. Added

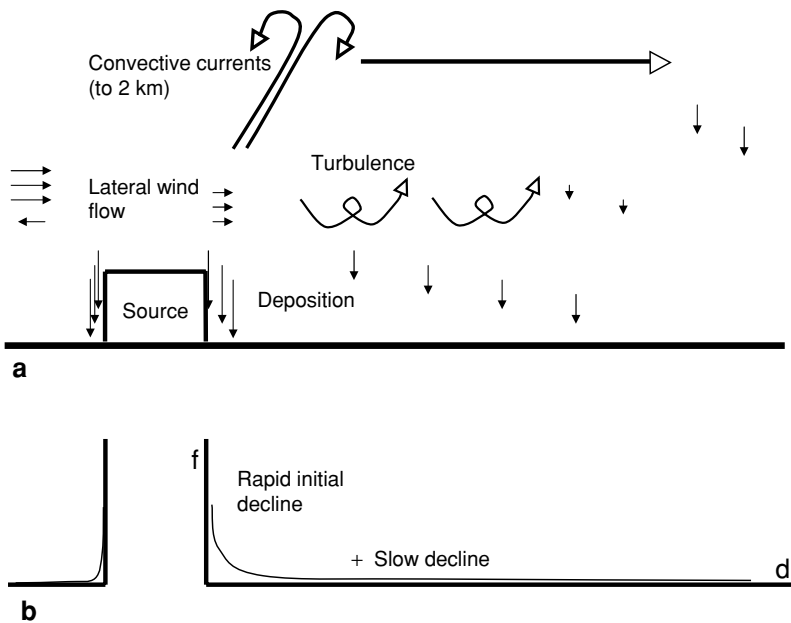


Figure 3.9 Wind-mediated pollen dispersal: (a) forces affecting dispersal, (b) effect on observed pollen dispersal with distance from source [f: (pollen) frequency; d: distance (from source field)].

to these factors are the convective currents that may lift pollen to various heights, including the surface of the atmospheric boundary layer during periods of strong convection. All of these processes combine to give a pollen dispersal curve that is essentially leptokurtic (Figure 3.9).

Pollen generally clears from the air overnight. Settling because of gravity is thought to be a main cause, with calmer air and a lack of convective forces assisting the process. Maize pollen is widely regarded as having a high settling rate, and its relatively large size certainly supports this assumption. However, deposition may be relatively less important as the forces during the middle of the day counteracting deposition may be much more effective, and impaction on surfaces a more important means of removing pollen from the air. The quantities of pollen depositing on land by all these processes are enormous: Hyde (1950) estimates the average fall of Poaceae pollen over the United Kingdom to be 20 million pollen grains $\text{m}^{-2} \text{y}^{-1}$.

3.5.2 Long-distance dispersal

Luna *et al.* (2001) investigated the scope for long-distance dispersal from maize in the United States. Knowing that wind was likely to be the only vector of significance, the authors measured the viability of pollen in ambient conditions and then determined that local wind speeds would mean that the theoretical maximum lateral distance travelled would be 32 km. They also observed cross-pollination from a 4000 m^3 area of maize containing a marker gene, and found detectable levels of cross-pollination at 200 m but not 300 m from the source. Dispersal and effective cross-pollination have also been recorded in the United Kingdom, where Weekes *et al.* (2003) have detected GM cross-pollination in farm-scale trials of maize occurring up to 630 m from the source.

The processes described in Figure 3.9 imply that some pollen will be taken distances that go well beyond the limits considered by experiments tracking a known type of pollen. Whether such pollen is likely to remain viable over these long distances has been a matter of debate. In an interesting series of experiments involving pollen sampling by impaction onto Petri dishes mounted in a device attached to the wing of a light aircraft, Brunet *et al.* (2003) monitored in the air up to and above the atmospheric boundary layer at 1.8 km, not just pollen concentration but the viability of that pollen. The convective currents during these experiments were lifting maize pollen to all heights with average concentrations of between 0.2 and 1.1 grains m^{-3} across the atmospheric boundary layer. Such values are low compared to the values recorded near maize fields on the ground, but are perhaps not much lower than terrestrial samples tens or hundreds of metres from maize fields. The viability of the trapped pollen varied with altitude: 40–50% near the ground to 5–10% above the atmospheric boundary layer. The high lateral wind flows often encountered at such altitudes mean that it is inevitable that a very low level of cross-pollination will occur over very long distances from sources of maize pollen, possibly even hundreds of kilometres. This effectively means that total isolation of GM maize by distance may not even be feasible on a regional or national basis.

3.5.3 *Local barriers, directionality and edge effects*

Barriers to airborne pollen such as hedges, trees and high crops are often thought of as possible modifiers of levels of gene flow from anemophilous crops. Understanding the processes should guide these considerations. For example, if convection is one major process of lifting pollen into the atmosphere, the value of barriers near ground level may be minimal. Of course, high and permeable barriers that filter out pollen by impaction may have the benefit of reducing pollen leaving or entering a field by a turbulent lateral airflow. However, if that barrier is impermeable and the airflow lifts over it, or if convective forces are dominant, such obstacles will be readily avoided.

Topography can have a strong effect on rates of pollen dispersal by air movement. Hills and valleys can channel airflow, leading to prevalent directions for air and so pollen movement, and local hot spots for wind strength may also modify levels of gene flow. Many crops flower during seasons when convective forces operate most strongly. Convection tends to be strongest during sunny weather when pollen release from cereals will be maximal. In addition to lifting air masses rapidly to high levels, convection will alter airflows at ground level, again influencing pollen dispersal. Patterns of airflow may be altered, and near coasts sea breezes will set up a locally predominant directional flow of surface air at right angles to the coast in seasons and at times of day when pollen release will be maximal.

Assuming that most of the extrinsic pollen arriving in a field arrives on a lateral flow of air rather than by settling from above, a strong edge effect on the upwind edge may be expected. To some extent, the crop itself will filter out extrinsic pollen as the airflow moves into the field and therefore reduce gene flow to internal parts of the field. However, the swamping of flowers in this field with local pollen (which will compete with extrinsic pollen) will also change across the field. Field edges are likely to receive less pollen from the field itself, enhancing the tendency for the edge to show higher levels of gene flow. Both factors, the arrival of extrinsic pollen and the reduction of intrinsic pollen available for flowers near the edges of fields, are likely to be greatly reduced on downwind edges of the field. Consequently, edge effects in fields of anemophilous crops are likely to be present and will show strong directionality.

3.6 Processes and patterns with insect-mediated pollination

3.6.1 *Functional groupings of pollinators*

Even straightforward features of the movements of many flower-seeking insects are unknown: what cues do they use to locate flowers? In the well-studied honeybee, the methods bees use to locate new resources may include the famous waggle dance, or they may rely more heavily on odour plumes as the main cue (Wenner, 2002). Bees can learn the salient features of the landscape they forage over: can other insects? These questions are important for the understanding of gene flow by insects.

Social bees, like many other insects, are restricted in their foraging range as they need to return to a nest. However, the aspects of their behaviour that affect

pollination have two unique features: the ability to transfer pollen from individual to individual, and a generally highly efficient exploitation of the resources within their foraging range, with communication between members of the colony. The patterns of movement in other insects are much less well understood. They may be seeking specific crops for floral reward, oviposition or herbivory, and may be using similar cues to those used by the social bees. Alternatively, they may be dispersive animals, finding crop fields by chance. It is clear that for many of the insects that visit crops such as oilseed rape, we know almost nothing about the distance, directionality and regularity of their flights. Moreover, since some species will have no nest to return to, they may often have the ability to move pollen over longer distances than the social bees. Solitary bees are known to be effective and efficient pollinators, and are at least seasonally tied to a nest site. They are however generally less abundant in agricultural crops than the social bees from the genera *Apis* and *Bombus*.

3.6.2 *Common processes: local dispersal*

Local dispersal of pollen by *Bombus* species is reasonably well understood. Heinrich (1979) investigated many aspects of bumblebee foraging. The fidelity to not only sites and patches but also the path taken through the area by a single bee on different days is striking. Thomson (1996) also described this trap-lining behaviour, and the patterns persisted when the array of plants in pots was rearranged. Dispersal distance, dispersal directionality and pollen shadows of bumblebees were investigated in natural stands of *Erythronium* by Thomson and Thomson (1989) using transplanted flowers of a variant pollen colour absent from the resident plants. Thomson and Thomson observed 40 flowers in the natural stand, and after only a single visit to the red pollen flower, about 100–900 red grains were found deposited on the other flowers, mostly on the first few flowers visited by the bumblebees. The mean distance of the movement of marked pollen by the bumblebees was between 1 and 16 m, and the maximum distance recorded in these 40-flower sequences was from 2 to 36 m. Each bumblebee tended to follow its own trajectory through the experimental area, giving rise to a clear directional component in the data. Pollen shadows were similarly investigated by Cresswell (1994) who measured the decline in carry-over with consecutive flower visits. A similar process is likely to occur with any insect, giving a high degree of carry-over across near-adjacent patches and a rapid decline at longer distances into the plot.

3.6.3 *Processes and patterns for social insects*

The revisiting of a small number of near-adjacent patches is the normal mode of behaviour for foraging honeybees. The constancy of bees to one particular flower type was noted by the ancients: ‘On each expedition the bee does not fly from a flower of one kind to a flower of another, but flies from one violet, say, to another violet, and never meddles with another flower until it has got back to the hive’ (Aristotle’s *History of Animals*, IX, 40, quoted in Proctor *et al.*, 1996). This



Figure 3.10 Opportunities for nest-mate mixing of pollen at the hive entrance.

constancy of the honeybee to flower species extends to a site constancy too. In a review, Levin and Kerster (1974) cited many studies that demonstrate foraging by individual honeybees often involves repeat visits to a particular site, with only limited movement beyond the patch while foraging. These observations, beginning with those of H. Müller in 1882, suggest that the scope for gene flow between patches by honeybees may be limited.

DeGrandi-Hoffman and Martin (1993) pinned clean bees to the entrances of bee hives and demonstrated that appreciable quantities of pollen can be transferred from bee to bee in the hive. The opportunities for bee-to-bee pollen spread can be clearly seen, even at hive entrances where bee density is often low compared to the combs inside the hive (Figure 3.10). Enhancement of nest-mate mixing to improve the efficiency of cross-pollination was explored by Hatjina *et al.* (1999). Simple devices were designed to brush pollen off bees at the hive entrance onto a variety of materials and a felt fabric was found to have the greatest effect (a 64% increase) in pollen richness on bees departing from the colony.

Ramsay *et al.* (1999, 2003) investigated nest-mate mixing by a combination of approaches. Pollen loads were collected from foragers returning to a colony; loads were broken open and pollen removed from an uncontaminated central portion was stained and examined. In the colony examined, more oilseed rape than other pollen types were coming in. All non-oilseed rape pollen loads examined contained some oilseed rape pollen, and pollen of all the entomophilous species that the colony was working was present across loads of other types. The colony was working one GM and several non-GM fields. Bees were collected emerging from the colony and applied to male sterile oilseed rape plants. The progeny seed was of mixed genotype from each bee, again suggesting extensive nest-mate mixing.

Trap-lining on a local scale, giving repeated revisits to plants in a set order through a mixed stand, was described previously as an example of a local process. Because bumblebees forage over wide areas, it is likely that this mode of foraging

will also operate on the landscape scale. If it can be shown that this does indeed take place, it could be a crucial element in the movement of pollen around the landscape. If this mode of behaviour is sufficiently common, and if insects do not become replete after visiting the first field, then the movement of fully pollen-charged bees from one field directly to the next could provide most of the cross-pollination that takes place between fields. Trap-lining extends beyond the social insects. The butterfly *Heliconius ethillia* has food plants from several genera that were often located 10 m or more apart in an area under study. This pollinator established 'trap-lines' which were patrolled with some regularity (Ehrlich & Gilbert, 1973, quoted in Levin & Kerster, 1974). Trap-lining also occurs on much larger scales. Janzen (1971) described the foraging behaviour of female euglossine bees of the neotropical lowlands; they also run trap-lines visiting scattered pollen sources over wide areas. Even some tropical hummingbirds go trap-lining, covering very large distances with their movements (Handel, 1983).

Figure 3.11 summarises the four ways in which bees may move pollen around the landscape. Scout bees, exploring the landscape, may make casual runs across the landscape with no real pattern. Foraging forces may switch their attention from one resource to another as the quality of the resource declines. Deliberate and repeated trap-lines may be worked by *Bombus* and some other bees. Finally, nest-mate mixing is undoubtedly a process that will mix pollen across the entire foraging range of the colony, particularly for bees with large colonies such as the honeybee.

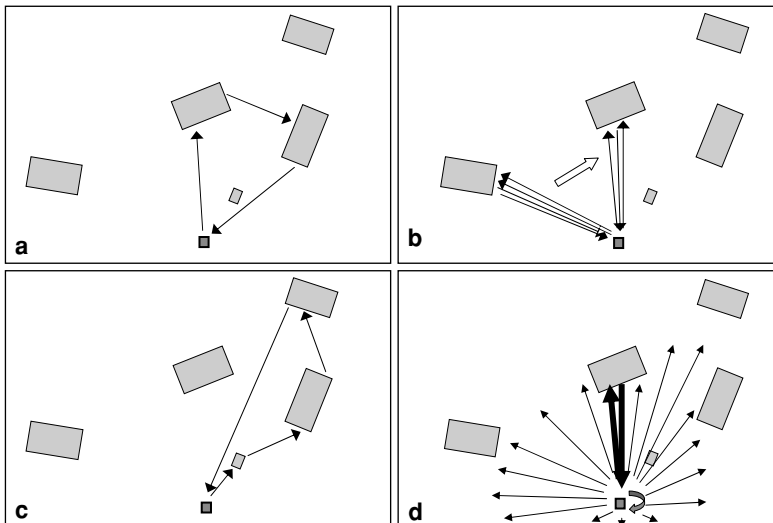


Figure 3.11 Routes and patterns of pollen dispersal by bees in an agricultural landscape: (a) scouting or wandering from crop to crop, (b) switch of a foraging force to a different crop, (c) trap-lining along a regular route and (d) pollen spread by nest-mate mixing.

3.6.4 Edge effects in recipient patches

Edge effects are predictable for both wind- and insect-mediated pollen dispersal. In the case of insects, the short hops taken in a foraging bout may take the insect across the boundaries of the field or plot. Also, new arrivals in fields have a tendency to settle in the first edge encountered. These events would tend to cause higher levels of gene flow near the field margin, and so a gradient will be set up moving in from the edge. In the studies of Fryxell (1956) in cotton and Ramsay *et al.* (2003) with oilseed rape, clear edge effects were noted, suggesting that these processes were in operation in these experiments. Such edge effects are not universal, however, and may even be reversed in special circumstances (Bond & Pope, 1974). In this case a difference in flowering time between the centre and edge of the field may have been responsible, but higher levels of gene flow at the edges of plots should be expected in most cases. As bee movement is insensitive to the direction of light winds, and bees and other insects may even sometimes orientate by flying up odour plumes, it is unlikely that, as with anemophilous crops, the upwind edge of a field would have higher levels of cross-pollination than other edges. Indeed, if nest-mate mixing is a primary means of generating cross-pollination between fields, the edge of the recipient field with the highest level of cross-pollination may not be the one facing the source field (Figures 3.9 and 3.12). However, cross-pollination by insects flying

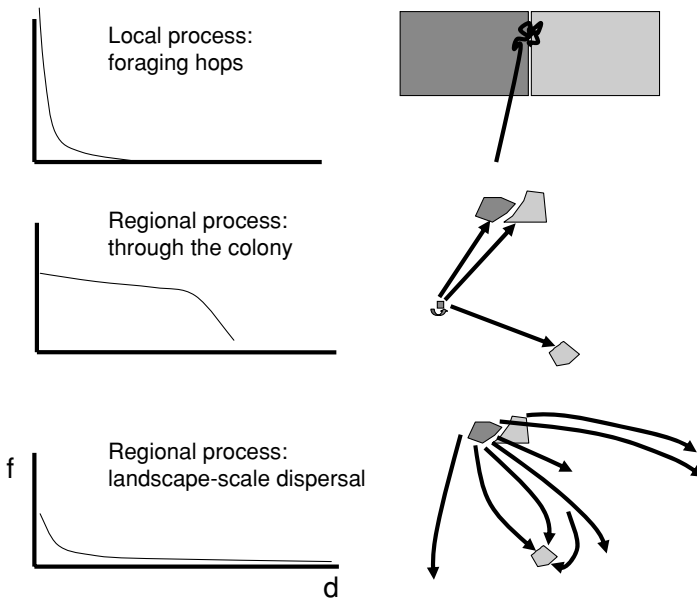


Figure 3.12 Simplified theoretical dispersal functions of the different components of insect-mediated pollen dispersal [f: (pollen) frequency; d: distance (from source field)].

directly from one field to another, such as trap-lining bumblebees, may cause higher levels near the edge facing the source.

3.6.5 Patchiness and pollinator behaviour

For a long time, it has been widely recognised that there is a relationship between plant spacing and bee flights (Handel, 1983). This was demonstrated most clearly by Levin and Kerster (1969). Among populations of nine species, the correlation between plant spacing mean and bee flight mean was 0.9. This appears to be common sense, in that bees are responsive to their environment and will readily fly further to visit new flowers when necessary, but prefer to visit close ones if they are available. These trends also apply to plants growing in patches. Bee flights between patches are undertaken readily: the metabolic cost of long-distance flights is outweighed by the advantages of more efficient foraging in desirable patches. Much of this decision-making process is undertaken at the colony level in honeybees with individuals selecting single patches to visit, whereas bumblebees may operate at the level of the individual, typically sampling from closely or widely spaced patches during a foraging bout. The outcome of this means of foraging is that the distance between patches, within ranges that may be several kilometres across, has little effect on rates of pollen transfer and gene flow. There is evidence from studies using molecular markers that, in tropical forest trees, fragmentation of habitat and the resultant increase in distance between isolated trees can increase the rate of gene flow by insect pollinators over long distances. In Honduras, insects pollinate *Swietenia* trees in a deforested landscape over distances exceeding the separation to the next fragment, even when this distance was 1 km (White *et al.*, 2002). Indeed, one isolated tree received most of its pollen over a distance greater than 4.5 km. Similar events were seen with the forest tree *Dinizia excelsa*, reported in the paper by Dick *et al.* (2003).

Stacy *et al.* (1996) reported that in undisturbed forests in Panama, most pollen movement occurred to the nearest neighbour when trees were clustered, but that as interplant distances increased, pollen flow to these isolated trees became less likely to come from the nearest neighbour. These effects were noted in pristine habitat, though fragmentation by deforestation can also change the spectrum of available pollinators and may add to any effect of the increased mean foraging distance of pollinators. For example, plants that require buzz pollination can be efficiently pollinated by native pollinators but are less likely to be pollinated by honeybees, which are often more abundant in managed habitats (Roubik, 1978).

Similar effects are noted in herbaceous plants. Ellstrand *et al.* (1978) found that outcrossing in wild *Helianthus annuus*, a predominantly bee-pollinated crop, was negatively correlated with plant density.

It should be noted that although these observations relate to pollinator behaviour in response to their environment, in wind-pollinated species the density-dependent alterations in gene flow can also operate. Bannister (1965), for example, has suggested that in dense stands *Pinus radiata* individuals may have potentially fewer

mates than in sparse populations. This may be because in dense stands there is more pollen delivered locally than is needed for full seed set, and so the overabundance of pollen from close neighbours effectively outcompetes the less frequent incoming pollen. The key consideration for isolated individuals of wind-pollinated species is whether the airborne pollen rain is sufficiently dense to effect full or partial seed set.

3.6.6 *Influence of landscape patterns on pollen dispersal*

Patchiness discussed above is one element affecting foraging behaviour and pollen dispersal. Fields can be considered as large patches, and their patchiness will affect bee flight. High density of fields and low competition for bee forage will encourage short flights; low density of good nectar and pollen sources across the landscape and higher levels of competition may encourage long foraging flights.

Beyond these aspects related to patchiness, other factors may influence the intensity of pollen dispersal and gene flow to particular sites. Wind direction and so the directionality of odour plumes will affect bees and other insects. Landscape features affect flight lines, with evidence that some bees use landscape features as markers for following routes (Osborne *et al.*, 1999). These aspects of bee behaviour will undoubtedly influence the rates of pollen dispersal experienced on a fine scale, perhaps regarding hot spots in single fields. However, there is evidence that on the larger scale, landscape features that greatly suppress gene flow are unlikely to be found. Ramsay *et al.* (2003) demonstrated that bees can locate resources out of sight and navigate towards them. Pollination of male sterile oilseed rape plants, shown by the authors to be mediated by insects, mostly bees, took place across belts of mature plantation forest 3 km deep and a further 2 km of arable land. Individuals of different *Bombus* species can be seen commuting to offshore islands and over high mountain passes, so it seems most unlikely that any such landscape feature can be regarded as an absolute barrier to gene flow.

3.7 **Modelling pollen dispersal based on vectors**

3.7.1 *General models*

A variety of modelling approaches have been used to address the uncertainties surrounding the pollen dispersal from fields of crops such as oilseed rape. The method for oilseed rape invoking individual dispersal within fields was proposed and later tested by Lavigne *et al.* (1998). This approach made no assumptions on the vectors involved. Other empirical approaches also do not involve the modelling of vectors and so will not be discussed further here, other than to point out that the predictive ability of any model that does not take into account the behaviour of the important vectors is likely to be low. A paper by Walklate *et al.* (2004) assumes that an atmospheric dispersal model can form the basis of explaining and modelling gene

flow in oilseed rape and, somewhat bizarrely, invokes a local function to model insect redistribution of pollen once the primary distribution by wind has been achieved.

Models for maize pollen dispersal are much more straightforward and can proceed making assumptions of airborne particulate dispersal. For example, Klein *et al.* (2003) have published a model that uses an individual dispersal function and incorporates attributes of Brownian motion, settling velocities, wind speed and turbulence. A further predictive model for an anemophilous species is that of Giddings (2000), which gives an indication of the possible rates of cross-pollination in *Lolium perenne* using a Gaussian plume model. The model predicted that a small conspecific population 1 km from a large source could be swamped with pollen from that source.

3.7.2 Modelling elements of bee behaviour

Cresswell (1994) quantified the decline in cross-pollination by a bumblebee charged with pollen as it foraged on a series of oilseed rape flowers. This component of the insect-mediated pollination in oilseed rape was elaborated on further by Cresswell *et al.* (2002), and an exponential power law function was derived that described the decline in the proportion of transgenic progeny as a bumblebee moves from flower to flower in a patch. Both honeybees and bumblebees appear to have similar paternity shadows (Cresswell *et al.*, 1995). This paternity shadow is one major influence on level of gene flow by bees. Levels of gene flow will also depend on the number of new visits made to the patch (and so the residence time in the population) and also the proportion of incoming insects that carry extrinsic pollen rather than those that have revisited the patch. As residence in a patch appears to be longer in bumblebees than in honeybees (Bateman 1947; Cresswell *et al.*, 2003), this aspect of the total level of gene flow contributed by these species may be greater for honeybees. However, fidelity to single sites is known to be very high for honeybees. This implies that if most workers are revisiting patches, the contribution of honeybees to gene flow will be low due to bee-to-bee pollen transfer in the hive and relatively rare switches of foraging sites by individual bees. *Bombus* foraging habits are much less well known, but trap-lining is described for some species where sequences of plants may be repeatedly revisited over periods of days or weeks. Observations of foraging on fields of oilseed rape (Ramsay & Thompson, unpublished) suggest that bumblebees can forage in a directional manner on a field scale. On this occasion but not in other seasons, most foragers, involving several species of *Bombus*, arrived at the east side of a field, proceeded foraging across the field westwards and left the far side of the field in the same direction. The factors setting up this directional flow of bees were not determined, but the observations suggest that direct field-to-field flights can be common in *Bombus*.

To model the various components of landscape-scale pollen dispersal, it may be necessary to break down the whole into several discrete components, each with their own variants for particular situations. An example of how this might be achieved is given in Figure 3.12, where local-scale events are separated from the regional-scale events.

3.8 Lessons for the management of gene flow from studies on vectors

3.8.1 *Effectiveness of isolation by distance*

Isolation by distance is hard to achieve. With any vector, local processes affecting gene flow decline rapidly and levels fall off quickly with distance, permitting most thresholds for crop purity to be met readily. However, the lower level of gene flow found from more regional processes is relatively insensitive to distance. As anemophilous species are liable to send pollen via convective currents to high altitudes, no distance can be specified at which no cross-pollination will occur. The low levels already detected on the ground at distances up to about 630 m from the source may represent the long tail of the dispersal function that may decline only very gradually with distance.

Similarly, entomophily brings with it the possibility of long-distance dispersal that given the efficiency with which pollinating insects can find stigmas, may continue in the right circumstances at relatively high levels for long distances. The most efficient insects for landscape-scale dispersal are certainly bees. From studies such as those of Goulson and Stout (2001) we know that bumblebees can forage over distances of several kilometres. Long-distance foraging also takes place in the honeybee. Eckert (1933) demonstrated that honeybees will carry sweet clover pollen from an irrigated area at least 8.5 miles (13.6 km) and possibly 10 miles (16.1 km) back to the hive. He also reported that in a favourable year, 1928, colonies 7 miles (11.2 km) from the nectar source produced an average daily weight gain approximately the same as colonies within the local irrigated area in the previous year, a year acknowledged by local bee-keepers to be a poor one for honey harvests.

3.8.2 *Managing seed and crop production systems*

Barriers to wind have been considered as tools to minimise cross-pollination in anemophilous crops. Meier-Bethke and Schiemann (2003) indicate that physical barriers to pollen flow are unlikely to be effective at the emitting field, but would be a useful measure at the receiving field. The possibility exists that lateral airflows would simply climb over barriers, taking their pollen grains with them and rendering the barrier ineffective for the purpose. Furthermore, convective airflows will completely avoid any barrier surrounding a field. This indicates that the measures that could be taken by farmers growing anemophilous GM crops to reduce gene flow to other fields in the region are unlikely to be effective. On the other hand, measures to ensure the purity of the production of seed for planting rather than crop production are feasible. In addition to extreme isolation by distance, the seed producer can manage purity by discarding edge strips. Choices can often be made for the location of fields for seed production, and factors such as predominant local wind direction, including temporary wind flows such as sea breezes, can be taken into account.

Where bees are deliberately introduced, it appears to be safe to assume that the carry-over of germinable pollen from previously pollinated crops is negligible. Kraai (1961) found that one overnight enclosure, the normal practice for the movement

of a colony for pollination purposes, was sufficient to bring down the levels of germinable pollen on bees in the hive to a very low level, as measured by seeking the inheritance of marker genes in the progeny. When honeybees are introduced into an area for pollination, they are normally more densely stocked than when sited for honey production. Overexploited localities may force bees to forage over a wider area, and so this factor should be taken into account when planning pollinator introduction to sites where gene flow ought to be minimised. It may even be feasible to manipulate pollinator populations to reduce pollen dispersal in some circumstances. Red mason bees (*Osmia rufa*) are reputed to forage over distances significantly less than honeybees and bumblebees, perhaps up to 1 km (Chris O'Toole, The Oxford Bee Company, personal communication, 2004). These bees collect pollen to provision their nests but do not collect appreciable quantities of nectar; so although stocking an area with this bee may reduce pollen gathering by other bees, they are unlikely to make the flowers unattractive to nectar gatherers.

Staniland *et al.* (2000) investigated the effect of oilseed rape borders around source fields. They appeared to have the ability to absorb pollen, and displayed low levels of gene flow beyond 10 m into the border. Although such an approach has the benefit that the farmer growing the GM type can adopt the measures, there is little evidence so far that the effect is also present at the receiving fields in the locality. Morris *et al.* (1994) compared barren zones with border strips and found that barren zones are relatively ineffective. These results are consistent with the behaviour of insects rapidly crossing these barren zones rather than diluting the pollen they carry by further flower visits as they leave the source of the pollen of interest.

Patterns of arrival of insects in fields can predict the patterns of edge effects likely to be seen. In casual observations, it can be seen that in some circumstances, bees fly into the centre of a plot, presumably revisiting a patch previously found to be rich in resources. Other bees can be seen arriving on the edge and foraging inwards into the field. Each mode of behaviour will impact on patterns of pollination seen, one giving a pronounced edge effect, and one not. The limited results from studies on edge effects so far concentrate on plots and fields close to a source, and whether the edge effect declines in insect-pollinated crops at greater distances from sources, as local processes give way to regional processes, remains to be seen. However, a male sterile plot of oilseed rape grown at the Scottish Crop Research Institute (Dundee) about 1 km from the nearest field also showed a pronounced edge effect, and this implies that the edges of fields will always carry more impurity than the centres, no matter how far from the source the field is sited (Cullen *et al.*, 2004). Both this study and the earlier one of Ramsay *et al.* (2003) noted more events at corners than at any edge, perhaps reflecting subtleties of the behaviour of pollinating insects. The data on edge effects, the causes of differences between different edges and the differences between edges and corners will all help perfect strategies to limit impurity by discarding the outer portions of seed production fields.

Understanding of landscape-scale gene flow has increased greatly in recent years, but there are still gaps in this knowledge. Several wind-mediated models exist that can, with a minimum of additional data, predict likely pollen dispersal of different

anemophilous crops. The component of the cross-pollination that is due to insects, dominant in many crops and present at a low level in many others, is much harder to model and will always present a complex picture. Bee-mediated pollination dominates in many insect-pollinated crops, and here there is a better understanding of local and regional processes. However, even the common and abundant *Bombus* species in many temperate regions is relatively poorly understood in regard to the factors that may influence their role in cross-pollination. Simple but crucial data on *Bombus* patterns of foraging on the landscape are missing: when presented with a choice of fields, do they regularly commute from one field to another, or are most movements to and from the colony, as in the honeybee? Other insects are even more poorly understood.

The challenges facing those involved in modelling gene flow in crops with mixed pollination systems are far from trivial. Different locations and different years may present very different mixes of insect pollinators, and each type of insect will have its own pattern of landscape and resource use. The behaviour of each insect will vary according to the competing available forage, and the requirements of each insect species and their activity will vary with season. Weather influences insect behaviour in complex ways: low or high temperature during foraging affects the overall activity of some insects more than others, and wind direction can have a dramatic effect depending on whether the insects are travelling with the wind, are using odour trails to locate plants or are following previously learned routes independent of the current wind direction. Distance will interact with all vectors in complex ways. Wind as a pollen vector may be less or more important near pollen sources, and weather may influence the distances insects are prepared to fly in certain conditions.

So, the plethora of studies in recent years measuring and understanding gene flow in a selection of crops that have been or may be commercialised as GM crops has given us a much more realistic view of the prospects for meeting certain purity thresholds, and a hint at the distances over which low levels of gene flow may be found. However, since our understanding of the role of many insect vectors and the interaction of their contribution with features of the landscape remains vague, our ability to predict gene flow for new crops and new situations remains very restricted. Bridging this gap is a major challenge for entomologists and modellers in the years ahead.

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4 Hybridisation – reproductive barriers to gene flow

A.J. Richards

4.1 The taxonomic context of hybrid formation

Only plants of economic importance are likely to undergo GM for the foreseeable future. Economically important crop plants are rarely native over most of the range in which they are grown commercially. Consequently, concerns that novel genes (i.e. transgenes) introduced into GM crops will move into the broader environment have centred on the possibility that this will occur by hybridisation between the GM crop and naturally occurring wild species. It is argued that the presence of certain transgenes that protect plants from herbivores, herbicides or pollutants, or confer other physiological advantages, might cause some naturally occurring plants to expand their ecological or geographical range, thereby upsetting delicate ecological balances (Raybould & Gray, 1994). Gene flow by hybridisation within an agricultural setting might enhance the competitiveness of existing weedy relatives and hence prove injurious to crop systems.

The assumption that transgenes can only move into the wider environment after hybridisation with naturally occurring species is in fact highly misleading. This is because most crop plants were themselves originally derived from ruderal species that possess many weedy characteristics and so have the capacity to invade vacant niches. Indeed, as ‘escapes’ from cultivation and as ‘volunteers’ within cultivated systems, crop plants can often become serious weeds in their own right, as is already true for oilseed rape (canola) *Brassica napa* subsp. *oleifera* in the United Kingdom. If such a crop were also to contain certain transgenes that enhance this capacity, then the crop may become aggressively weedy in open environments, including those of other crop systems (Raybould & Gray, 1993). In that sense, when any crop possesses GM-derived attributes, it is probable that transgenes will readily move from the bounds of agriculture into the wider environment.

The fact that most crop plants have weedy characteristics, and were derived from weeds, means that the related species with which GM crops are most able to exchange transgenes by hybridisation are usually weedy species themselves. It follows that the habitats most likely to be exposed to invasion by wild and cultivated transgenic plants will be open, agricultural land, weedy and cultivated sites. Although still a cause for concern, the probability that closed natural and semi-natural habitats of conservation value will suffer from the ramifications of transgene movement is, by comparison, very much reduced (Gray & Raybould, 1998).

When assessing the risks of such outcomes, it is clearly pertinent to ask, ‘How likely is it that hybridisation might occur between crop plants and wild relatives?’ Interspecific hybridisation is hugely important in most higher plant taxa and is

found throughout most major plant taxonomic groups, life forms and biomes. To most evolutionary scientists who are unfamiliar with the taxonomy and population genetics of plants, this often comes as an unwelcome surprise. Most definitions of a species, and speciation, have been published by zoologists, and consequently use mating isolation as the main criterion. If such definitions were rigorously applied to higher plants, relatively few species, perhaps as few as 30% of the British and Irish native flora for instance, would survive the ensuing reclassification. Clearly, botanists have chosen to adopt different philosophies from zoologists if the species concept is to have any relevance to plants.

The substantive differences between plants and animals in the means by which speciation occurs can be largely attributed to animal behaviour. Most animals generally use senses to choose their mates (sight, smell, sound, and even touch and taste). They have developed extremely complex behavioural systems that have often evolved to optimise mate quality/fitness. The mechanisms that operate these systems have lent themselves to 'hijacking' by positive feedback and other forms of sexual selection, which have in turn encouraged the diversification of accurate mate recognition systems, which has driven animal speciation. In addition, mechanical barriers to hybridisation can also be important, for instance in many insect groups such as the Lepidoptera. Equally important, animals possess immunological systems so that foreign (non-conspecific) sperm or internally carried hybrid embryos are likely to be rejected within the female reproductive tract.

In strict contrast, plants are passive maters and lack immunological systems. As a consequence, the number of types of isolation barriers that plants can call upon to maintain species integrity is much smaller than for animals. It follows that isolation between related plant species is much more often temporal, ethological, ecological or geographical than is typical for animals. It is axiomatic that plant demes that have become sufficiently distinct to become recognised as species were at one stage sufficiently isolated for disruptive selection or chance to promote their genetic divergence uninterrupted by hybridisation. Nevertheless, when environmental circumstances change, pre-pollination barriers that have kept plant ecospecies separate are much more easily disrupted than are the behavioural, mechanical or chemical barriers typical of most animals. As a consequence, hybridisation amongst plants occurs readily after environmental disturbance, for instance by the actions of man.

The intervention of man has frequently disrupted the barriers that isolate plant species, most commonly by accidentally introducing species into non-native areas or intentionally into horticultural or agricultural systems. The role played by mankind in aiding and abetting the transportation of species outside their native areas is at least as old as agriculture itself, and indeed much of contemporary agriculture now depends on crop species that evolved as man unintentionally brought their parents into contact. Man has also hybridised habitats, allowing ecospecies to come into close contact, although they had been previously isolated in different environments. Frequently, maladapted hybrids have been granted a brief window of opportunity by the evanescent open habitats that man has fleetingly created.

Additionally, climate change has shifted the seasons, allowing the flowering times of species to overlap much more than was previously the case. Pollinators have taken advantage of exotic sources of nectar and pollen that they transport to the stigmas of their habitual symbiote. Wherever we examine the effect of mankind on the natural world, we encounter plant hybridisation. Thus, we can suppose there is a considerable potential for transgenes in crops to move by gene flow into non-crop and native species, and this forms the subject of this chapter.

4.1.1 *What is a (crop) species?*

The word 'hybridisation' can be applied to sexual reproduction between any two genetically distinct individuals. Thus, it is often necessary to qualify the term, as in interspecific hybridisation (see above). By definition, interspecific hybridisation occurs between two individuals classified into separate species, but as we have seen, it is not as easy to define a plant species as an animal species. There is a real danger of circularity, because the ease with which two taxa can hybridise might be considered the main criterion for deciding whether the taxa should be recognised at the specific rank. Typically, interspecific hybridisation in plants only occurs with difficulty, or if hybrids do occur, they are often sterile. To some extent, however, these observations are merely semantic consequences of the definition of a species. When there are no barriers to hybridisation between two taxa, it becomes very difficult to distinguish between populations that are actively hybridising with each other, and those that are merely poorly differentiated.

It has been irreverently said that there may be as many definitions of a plant species as there are plant taxonomists. More relevantly perhaps, there should be as many definitions of a species as there are critical groups of plant taxa, because the reason why a particular group of plants does not lend itself to easy classification differs in every case. For any general concept of the plant species to remain workable, it has to be left deliberately vague. Fortunately, our considerations here are limited to the possibility of hybridisation between a crop plant and a wild relative, which will often (although by no means always) represent its progenitor. In this context, the rank at which the two taxa are traditionally separated becomes relatively unimportant. In some cases, centuries of intensive breeding have modified the crop plant appearance to such an extent that it is no longer obvious that the crop and its ancestor(s) have only diverged from one another during the short period of evolutionary time since the crop was first domesticated. On at least one occasion, the morphological divergence between a crop species and its very recent wild ancestor is so great that they have been separated at generic level. Maize, *Zea mays*, is not known as a wild plant and seems to have been developed by man directly from teosinte, *Euchlaena mexicana*, with which it is interfertile (although the latter is now often called *Zea mexicana*, or even *Zea mays* subsp. *mexicana*). Unexpectedly, maize is not only interfertile with its presumed ancestor, but hybrids have even been created with such distant relatives as various wheat species (*Triticum* spp.), barley (*Hordeum vulgare*) and rye (*Secale cereale*) (Laurie *et al.*, 1990).

The distinction between a crop plant and its progenitor has more often been made at specific rank, and so rice (*Oryza sativa*) and potato (*Solanum tuberosum*) are amongst many other familiar examples of crop plant species not known in a wild state under that binomial name. Indeed, it seems probable that potatoes did not acquire the characteristics now considered typical of *S. tuberosum* until after they were introduced into Europe from South America (Simmons, 1976).

The degree by which the crop plant and its wild relative have been separated taxonomically probably reflects pragmatism, individual taste and historical accident as much as any measurable scientific reality. For instance, beet, beetroot and mangold (*Beta vulgaris* subsp. *vulgaris*) differ substantially from wild sea beet (*Beta vulgaris* subsp. *maritima*) in many characteristics, to an extent that might have warranted specific recognition for the crop varieties in other genera. However, most modern treatments only recognise these variants at the subspecific rank, perhaps because there is a long recorded history of hybridisation between sea beet and crop beets in the wild (Tufto *et al.*, 1998). When subspecific rank for the crop plant has been considered appropriate, the crop variety takes the type name on some occasions (*Pastinaca sativa* subsp. *sativa*, cultivated parsnip) and the wild variety does so on others (*Daucus carota* subsp. *carota*, wild carrot).

In other crops such as *Brassica oleracea*, it can be argued that the great diversity of cultivated varieties (cauliflower, sprouts, broccoli, calabrese, kale, cabbage), and the considerable variability of so-called wild populations that may have both originated from and latterly backcrossed to cultivated populations, has mitigated against recognition at more than varietal rank (Mitchell & Richards, 1979). In other cases still, there has been no taxonomic recognition of cultivated varieties at all, although these now differ substantially from the wild parent (for instance, *Rubus idaeus*, raspberry, or *Ribes nigrum*, blackcurrant).

Many crop plants were not derived directly from a single wild progenitor, but have a hybrid, usually of allopolyploid origin. Often, neither parent occurs over much of the range of the crop. Perhaps the most famous example is allohexaploid wheat, *Triticum aestivum*, which is derived from three parents classified into two genera, all confined to western Asia where wheat originated 8000 years ago (Zohary & Feldman, 1962). Alternatively, one of the parents of the crop may be present and the other absent. For instance, one of the parents of white clover (*Trifolium repens*), *Trifolium nigrescens*, is occasionally sympatric with crop clover in southern Europe and is itself sometimes used as a crop, but the other parent (*Trifolium uniflorum*) has a strictly Mediterranean coastal distribution (Badr *et al.*, 2002). Similarly, oilseed rape (canola), *Brassica napus*, is an allotetraploid crop that is more often found in the company of one parent (*B. rapa*) than the other (*B. oleracea*).

More importantly perhaps, backcross hybrids between an allopolyploid and one of its diploid progenitors are usually triploid and therefore mostly or totally sterile. This illustrates a major conclusion of this chapter, that hybridisation and the potential for gene flow are not the same thing. Many hybrids are sterile, or nearly so, and so transgenes will rarely, if ever, be able to enter the gene pool of a wild relative by introgression because of the inability of the hybrid to backcross.

We can conclude that the classificatory rank that separates a crop species from its wild progenitor(s) or other relatives is too often an accident of whim or of history for it to be used as any sort of guide as to the likelihood that fertile hybrids can be formed between them. Rather, in order to assess the probability of hybridisation between crops and wild plants, it is necessary to concentrate on other indicators such as proximity of the crop plant to the wild plant, the degree of overlap in flowering times, the mating system of each potential parent, the distance of pollen flow, interspecific incompatibility, seedling establishment and F_1 fertility (Daniell, 2002).

4.2 Reproductive isolation

The concepts of hybridisation and reproductive isolation are antithetic, and hence the possibility of hybridisation between two individuals depends directly on the extent to which they are reproductively isolated.

It is likewise important to distinguish between the potential for hybridisation (for instance in a garden or experimental glasshouse) and the likelihood that hybridisation may occur in the field autonomously, without the direct intervention of man. In cultivation experiments, taxa that are normally separated by habitat preference or geographical range can be brought together with ease. Similarly, if two potential parents normally flower at different seasons, flowering time can be manipulated by the adjustment of day length or temperature, or pollen can be desiccated and stored. Alternatively, if cross-pollination is prevented by pollinator behaviour or habitual autogamy, crosses can be achieved manually under experimental conditions. If an embryo is formed, but its development fails to align with that of the endosperm, or the embryo fails to develop in a genetically foreign environment (seed incompatibility), embryos can sometimes be rescued and developed in culture (Shinoda & Murata, 2003, provide a striking example). Our gardens are peopled by a multitude of often complex hybrids, most of which would never have occurred in nature. Thus, it is even important to distinguish between the ability to form hybrids under experimentation and the capacity to do so naturally. In the present context, I assume that GM crops are grown commercially on a field scale, so our considerations can be limited to the likelihood that hybridisation will occur outside the garden, in nature, without man's direct intervention.

4.2.1 *Potential for hybridisation*

It is useful to consider how the potential for interspecific hybridisation varies between different genera and families. Often, it is possible to create artificial hybrids between surprisingly disparate parents, even those classified in different genera, particularly if pre-pollination mechanisms have played a significant role in the speciation process. In cultivation, the possibilities for hybridisation in families with complex flowers and sophisticated pollination mechanisms such as the Orchidaceae (in which more than 20 000 hybrids are known) or the Cactaceae seem endless. Equally, in large,

widespread genera where geology and glaciation have isolated many populations on biological islands, few barriers to hybridisation seem to exist once the allopatric species are exposed to each other in the garden. *Rhododendron*, *Lilium* and *Rosa* are familiar examples of genera in which a great number of rather fertile wide hybrids have been produced. Major differences between species in genome structure and distribution between chromosomes may limit the fertility of these hybrids, which is nevertheless often surprisingly high. More typically perhaps, the potential for hybridisation between species classified within a subgeneric taxon (e.g. a section) may be considerable, but intersectional hybrids rarely if ever occur in the genus (e.g. *Primula*, *Carex* or *Saxifraga*). This argument can be circular, however, because the potential for hybridisation has often been used when sectional limits are set. In genera in which many hybrids occur in cultivation, reproductive isolation between species in the wild usually occurs before pollination, as the result of spatial, ecological, phenological or anthecological barriers. However, there are other large genera in which even intrasectional hybrids are rarely encountered. *Crocus* and *Fritillaria* are two large and popular genera grown in large collections (>100 species each in cultivation), but fewer than 10 hybrids are probably known in each, even in the garden environment.

Very little seems to be understood about why the potential for interspecific hybridisation varies so greatly between groups. Owens and Bennett (1998) have identified some indicators that may predispose distantly related taxa towards wide hybridisation:

- no self-incompatibility in at least the maternal parent
- similar amounts of nuclear DNA in both parents
- polyploidy in at least one parent
- individual genotype of parents

Where sympatric speciation has been triggered by minor floral changes in groups with complex pollination such as the Orchidaceae, it seems that speciation originally occurred in the absence of other isolating mechanisms, and so the potential for hybridisation persists. One might also argue that plants with complex pollination systems that have been placed in different genera or tribes because of their greatly divergent floral morphology might nevertheless be sufficiently closely related to form hybrids. It is also interesting that families such as the Orchidaceae, Asclepiadaceae, Araceae and Cactaceae are renowned for their complex pollination but are rarely self-incompatible.

4.2.2 *Wallace Effect*

Alfred Russel Wallace hypothesised that sympatric ecospeciation will favour the development of reproductive barriers between the differentiating demes (Wallace Effect, Grant, 1966a). Wallace argued that in one of the divergent populations an individual that possessed a heritable characteristic preventing it from crossing with the other population would be favoured over its competitors, as its offspring would

show advantageous, parental attributes, rather than maladapted, intermediate hybrid features. Therefore, we might expect the products of sympatric speciation to be more often reproductively isolated compared with allopatric species, much as Grant found in *Gilia*. This might explain the high levels of interfertility found in gardens between predominantly allopatric species in genera such as *Rhododendron*. However, such an explanation completely fails to explain the rarity of hybrids in other genera that also exhibit widespread allopatric speciation with many local neo-endemics, such as *Crocus*, *Colchicum* and *Fritillaria*. In these cases, it is possible that isolation has occurred through post-pollination, prezygotic mechanisms such as pollen and pollen-tube incompatibility.

It is convenient to classify reproductive barriers between plants on the basis of when in the reproductive cycle the process fails. The following categories are readily defined:

- Pre-pollination (spatial or temporal isolation)
- Post-pollination, prezygotic (interspecific incompatibility)
- Post-zygotic endosperm–embryo balance seed incompatibility
- Post-zygotic F₁ embryo inviability
- F₁ sterility

The Wallace Effect is most often exhibited in traits conveying pre-pollination isolation such as flowering time, or floral characteristics such as colour, shape, timing or pollinator reward. Species isolated in this way have the potential for hybridisation in the garden, although less so in nature. However, there are now several examples in which interspecific incompatibility has apparently been selected for between sympatric species, while related allopatric species are not cross-incompatible. The original classical example of *Gilia* (Grant, 1966a,b) is perhaps the best known.

In contrast, cytogenetic features such as polyploidy, translocations and inversions that vary between the diverging demes tend to render the F₁ hybrid sterile, but they do not prevent the creation of the supposedly maladapted hybrid in the first place. In comparison to pre-pollination mechanisms, isolation mechanisms that are manifest through F₁ sterility waste precious maternal resource on hybrid embryos, so they are much less likely to isolate a successful new species. Nevertheless, Vandijk and Bijlsma (1994) argued that when cytodemes that form sterile hybrids are sympatric, they should evolve different flowering times to prevent wasteful hybridisation.

4.2.3 Pre-pollination isolation

Pre-pollination breeding barriers are of exceptional importance in the maintenance of species integrity in nature, but they mostly fail as soon as related species are brought into cultivation. Also, unwittingly, mankind is greatly increasing the opportunities for hybridisation in nature by bringing into contact related species that were formerly allopatric, by modifying habitats so that ecospecies meet and by creating open habitats with reduced competition.

4.2.3.1 Distance

The commonest cause for failure of pollination is distance, caused by the ecological or geographical separation of taxa. Whether mediated by animals, wind or water, the pattern of pollen dispersal from a given source is almost invariably leptokurtic, and so the likelihood that a pollen grain from that source will reach a given stigma will obey the inverse square law when depending on distance (Richards, 1997). In general, pollen that is habitually dispersed by wind tends to travel further on average than that dispersed by insects, not least because individuals of wind-dispersed species tend to be larger and dominant in the habitat.

It is not always obvious whether wind or insects form the main agent of pollen travel. For instance, most pollen in oilseed rape (canola, *Brassica napus* subsp. *oleifera*) crop-stands is dispersed mechanically or by wind, although the flowers might be expected to be entomophilous, being showy, nectariferous and with somewhat sticky pollen. In the centre of dense rape stands, pollinators may be scarce, pollinator travel is very short, and physical contact between congeners is very frequent. It seems likely that marginal or isolated plants will more often be pollinated by bees, although this logic seems never to have been tested (but see Chapter 3).

For wind-pollinated crops, plot or population size, the exposure of the site, the height of the pollen source above ground and above surrounding vegetation, local topography and physical barriers to free pollen flow will all strongly influence wind dispersal of pollen. Most schedules of pollen dispersal have not examined deposition on stigmas, but the receptivity, size, position, distribution, pattern and density of potentially cross-fertile stigmas are as important to pollen-mediated gene flow as are dispersal schedules. It is more important to arrive than to travel, hopefully.

The very nature of leptokurtic travel means that most journeys are made over very short distances; distant events become rare, but very distant events are nevertheless not unknown. Occasionally, wind-dispersed grains will get carried by a gust above surrounding vegetation, lifted in a thermal, even to the jet-stream, and so its potential dispersal range becomes effectively infinite. Pine pollen is regularly trapped 3000 km from land. Although foraging bees regularly self-groom, some pollen grains remain, and so when the bee embarks on 'escape flights' to a new patch or the hive, some grains may travel more than 1 km in distance. The remote possibility that pollen grains from a GM crop may very occasionally achieve fertilisations over extreme distances clearly has major implications for hybridisation with wild species. To become established, a dominant advantageous transgene theoretically only has to arise in a population once.

When we consider the likelihood that crop pollen carrying transgenes might reach the stigma of a wild and potentially interfertile relative, then a series of additional factors become important including agronomic practices, land-use patterns and weed ecology. When herbicides are used within conventional crops, they are unlikely to kill weedy close relatives, and so wild radish *Raphanus sativus*, *B. rapa* or mustard *Sinapis alba* could conceivably occur as weeds in stands of *B. napus* with which they are potentially interfertile. In practice, pre-emergence application of herbicides, crop rotation and competition from the crop severely limits the extent

of such infestations, although they do occur. In the case of transgenes conferring herbicide tolerance, current emphasis is on the introduction of transgenes providing tolerance against broad action herbicides such as glyphosate or glufosinate ammonia. In these cases, unlike conventional herbicide application regimes, all plants other than the crop are targeted by the herbicide, including the coincident weedy relatives. Clearly, in such instances, the movement of the transgene into the weedy relative would provide it with selective advantage at least during part of the crop rotation.

Although most crops are planted in dense, two-dimensional arrays (fields), weeds related to the crop are much more likely to be distributed as weeds in the field or around crops in scattered sublinear populations, on field headlands, along lanes and access routes, or in the case of *B. rapa* subsp. *campestris*, along the banks of major rivers (Wilkinson *et al.*, 2000). Such distributions have major implications for the likelihood of hybridisation, and they make it possible to predict zones of contact between two species where hybridisation could potentially occur. This can lead to risk assessments with regard to the likelihood of hybridisation. Relatively few individuals of each species are likely to occur in close contact, and gene flow within the wild relative will often be one-dimensional rather than two-dimensional. However, the possibility of distant, hybridisation events persists. Wilkinson *et al.* (2003) estimate that as many as 50 000 hybrids between crop *B. napus* and wild *B. rapa* might occur annually across the United Kingdom, of which 6000 were predicted to result from long-range pollinations.

4.2.3.2 Temporal isolation (phenology)

Many examples exist of two interfertile ecospecies being at least partially separated by flowering time as well as habitat, although few studies have quantified the relative importance of flowering time in maintaining species isolation compared with habitat preference, or differences in pollinators. Some examples of phenologically divergent pairs of ecospecies that may be familiar to students of the flora of the British Isles are listed in Table 4.1.

Flowering time is at least partially under polygenic control, and a large number of genes have been identified that influence the interaction of the timing of floral initiation in the context of environmental triggers, such as day length and light quality, and physiological parameters, such as biomass and stored resource (Doyle *et al.*, 2002). In seasonal climates, early flowering genotypes are expected to

Table 4.1 Pairs of closely related plant species from the British Isles with divergent flowering periods

Earlier species	Habitat	Later species	Habitat	Difference in time of peak flowering
<i>Primula vulgaris</i>	Woodland	<i>P. veris</i>	Grassland	3 weeks
<i>Geum rivale</i>	Damp, open	<i>G. urbanum</i>	Dry, shaded	4 weeks
<i>Silene dioica</i>	Woodland	<i>S. latifolia</i>	Ruderal (adventive)	5 weeks
<i>Dactylorhiza purpurella</i>	Damp, open	<i>D. fuchsii</i>	Drier sites	3 weeks

respond to short days, or shorter days, in comparison with later flowering genotypes. Interestingly however, in many monocarpic species, floral initiation only operates in larger individuals. In non-seasonal climates, floral initiation is often triggered by apparently insignificant clues, for instance a few days without rain (Whitmore, 1984).

When it is possible to manipulate flowering times experimentally, so that crosses are made between early flowering and late flowering taxa, hybrids typically flower at times intermediate between those typical of their parents. Casual observations of most populations of variable, outcrossing species show that some variation in flowering time occurs. In general, individuals that flower at peak flowering season will probably be fittest by maximising the diversity of conspecific potential mating partners and will be receiving the greatest number of visits from pollinators. Although this should tend to lower the variance in flowering time through stabilising selection, competition between males for early occupation of stigmatic sites may favour early flowering plants, particularly in protogynous, dioecious and gynodioecious species (Epperson & Clegg, 1987). However, in many species, matings that occur late in the season often give rise to more numerous, better provisioned seeds. Such disruptive pressures may encourage the evolution of a greater variation in flowering time within a species and so provide scope for overlap of flowering season with relatives. On the other hand, where genetic isolation is advantageous, newly evolving ecotypes may take advantage of variation in flowering time to commandeer phenologically distinctive genotypes. In at least three metal-tolerant races (*Agrostis capillaris* and *Anthoxanthum odoratum*, McNeilly & Antonovics, 1968, and *Silene vulgaris*, Brooker, 1963), tolerant genotypes have been shown to flower significantly earlier than the surrounding non-tolerant populations.

Intensive studies on hybridisation in Pacific Coast *Iris* species have shown that relatively small differences in flowering time provide significant isolation between the few-flowered, highly protandrous *Iris chrysophylla* and *Iris tenax*, but in the many-flowered, relatively homogamous *Iris douglasiana* and *Iris innominata*, differences in flowering time are less important than habitat choice in providing genetic isolation (Young, 1996).

4.2.3.3 Ethological (pollinator) preference

Interfertile ecospecies are often isolated in the field by visitation from different flower pollinators, even when they grow sympatrically. The causes of such isolation between related species can be spectacular, as between the Californian *Mimulus cardinalis* (red flowered, bird visited) and *Mimulus lewisii* (white flowered, moth visited) (Nobs, 1954) or the various red and white tube-flowered Malesian rhododendrons (Richards, 1997). Perhaps the best example of all was furnished by Grant (1952) by another sympatric Californian pair, the red-flowered short-spurred *Aquilegia formosa* and the long-spurred yellow and white *Aquilegia pubescens*, the latter pollinated by hawkmoths (sphingids) and the former by hummingbirds. All such pairs tend to be very interfertile when crossed artificially, and these three examples have given rise to beautiful races of vigorous and fertile garden hybrids.

In other instances, the isolation seems to be just as strong, but to human eyes the causes of that isolation seem much less obvious. *Tellima grandiflora* and *Tolmeia menziesii* are both woodland members of the Saxifragaceae from northwest America with rather tall racemes of somewhat inconspicuous greenish brown flowers. Although they often grow together and they are not isolated by flowering time, hybrids nevertheless rarely occur. Weiblen and Brehm (1996) discovered that this isolation has several causes, but the fact that the outcrosser *Tolmeia* is nectariferous and visited by *Bombus* bees, whereas the mostly selfed *Tellima* is beetle pollinated, does much to preserve their genetic integrity.

4.2.4 Post-pollination, prezygotic isolation (interspecific incompatibility)

Isolation mechanisms that act between pollination and fertilisation following pollination by a foreign (different species) pollen grain have been known as 'interspecific incompatibility'. These mechanisms may involve the inhibition of pollen germination, failure of pollen tube growth or failure of egg cell fertilisation. When conspecific pollen competes with foreign pollen on a stigma or in a style, it may often grow faster than the foreign grains. For example, few hybrid embryos are formed when *Brassica campestris* pollen is challenged by *B. napus* pollen in *B. napus* styles (Hauser *et al.*, 1997). Conversely however, *B. napus* pollen is characteristically aggressive on *B. campestris* styles.

Interspecific incompatibility may result from the failure of the stigma to recognise (or to adhere to) a foreign grain, or from the interaction of intraspecific self-incompatibility systems between male and female parents in the interspecific cross. This might explain in part why the potential for hybridisation is low in certain genera, such as *Crocus* or *Fritillaria*, which typically have gametophytic self-incompatibility (GSI). Chichiricco (1996) showed that the failure of pollen tubes both after self-pollinations (late-acting self-incompatibility) and wide interspecific crosses in *Crocus* takes place within the transmitting tissue of the ovary, suggesting that a similar mechanism operates in both. Also, a comparison of the function of self-incompatibility in *Ulmus americana* and *Ulmus pumila*, and of interspecific incompatibility between these two species, strongly suggested that elements of the self-incompatibility mechanisms were involved in the cross-incompatibility. In both cases, pollen tubes failed to penetrate stigmatic papillae that contained highly fluorescent apically positioned plugs of callose, which apparently acted as physical blocks to stigmatic penetration in a manner reminiscent of sporophytic self-incompatibility (SSI) in the Brassicaceae (Bob *et al.*, 1986). Nevertheless, the site within the gynoecium where interspecific incompatibility is expressed is not predictable, even within a genus. Using *Pennisetum typhoides* as a mother, it was found that the pollen tubes of *Pennisetum cenchroides* reached the ovary but then failed, whilst those of four other species were inhibited in the style, and the pollen tubes of *Pennisetum polystachyon* and *Pennisetum pedicellatum* were unable even to penetrate *P. typhoides* stigmatic tissue (Mohindra & Minocha, 1991). Such results suggest that interspecific incompatibility is often not merely a by-product of intraspecific self-incompatibility, but may sometimes have a different basis as well.

Table 4.2 Site of pollen tube failure within the gynoecium in a series of interspecific crosses between *Primula* species

	Failure to germinate	Inhibition at stigmatic penetration	Inhibition in stigma	Inhibition in style	Inhibition at ovary	% seed set
<i>P. farinosa</i> × <i>P. modesta</i>						
P × T	30	9	9	35	17	10
T × P	8	0	0	46	46	0.6
<i>P. modesta</i> × <i>P. farinosa</i>						
P × T	21	12	0	0	67	0
T × P	3.5	0	0	0	96.5	0
<i>P. farinosa</i> × <i>P. modesta</i>						
P × P	36.5	41	16.5	1	5	0
T × T	100	0	0	0	0	0
<i>P. modesta</i> × <i>P. farinosa</i>						
P × P	69	18	11.5	2.5	4.5	0
T × T	98	2	0	0	0	0

Heterologous site recognition may also operate in diallelic incompatibility systems in heteromorphic genera such as *Primula*. In legitimate crosses (pin × thrum or thrum × pin) between the European *Primula farinosa* and the Japanese *Primula modesta*, pollen tubes are inhibited at a much lower position in the style than those resulting from illegitimate (pin × pin or thrum × thrum) crosses (Table 4.2). This suggests that interspecific incompatibility has a quite different mode of operation compared with intraspecific incompatibility in this group. Table 4.2 lists the percentage of tubes that are on average ($n = 5$) inhibited at various positions in the gynoecium after legitimate and illegitimate interspecific reciprocal crosses (P = pin flower, T = thrum flower) (Wedderburn & Richards, 1992).

Of particular interest is the mode of interspecific incompatibility after crosses between *Populus deltoides* and *Populus alba* (Rougier *et al.*, 1992). As members of the only large wholly dioecious family in the flowering plants (Salicaceae), poplars have no close relatives that are self-incompatible, and so the function of interspecific cross-incompatibility is unlikely to originate from self-incompatibility. As for *P. farinosa* × *P. modesta*, most *Populus* cross-incompatible tubes are arrested at the base of the style, near the ovary, perhaps because they become enveloped in a callose sheath in a manner very unlike the operation of most GSI systems (Richards, 1997).

4.2.4.1 Unilateral incompatibility

These observations from plants without GSI suggest that gynoecial interspecific cross-incompatibility often involves novel mechanisms not directly implicated in self-incompatibility. However, one of the most striking generalisations that can be made about the fate of interspecific crosses suggests otherwise. When pollinations are made between species with GSI and self-fertile relatives, it is invariably found that the self-fertile species accepts the pollen from the self-incompatible father, but when the potential mother is self-incompatible, the reciprocal cross cannot be made. This phenomenon is known as ‘unilateral incompatibility’ (Lewis & Crowe,

1958). The correspondence between the self-incompatibility system and the pattern of cross-incompatibility is such that the conclusion that the stylar-part incompatibility of the GSI system is directly implicated in the latter is compelling, as indicated by rigorous and far-ranging analytical surveys (Trognitz & Schmiediche, 1993; Hiscock *et al.*, 1998). It has been suggested that when self-incompatible and self-compatible relatives coexist, the former might benefit from this unilateral asymmetry in interfertility (Harder *et al.*, 1993). However, other studies have queried whether unilateral incompatibility involves the self-incompatibility mechanism at all (Liedl *et al.*, 1996). Interspecific crosses between gametophytically self-incompatible *Lycopersion pennellii* and self-compatible *Lycopersion esculentum* fail in the top part of the style although intraspecific self-incompatible selfs fail at a later stage, causing the authors to coin the term 'unilateral incongruity' in which interspecific incompatibility can be differentiated from intraspecific incompatibility. In any case, it seems that unilateral incompatibility is restricted to pairs of species in which one employs GSI as a mechanism to promote outcrossing. When we made reciprocal crosses between a heterostylous outcrossing species *P. farinosa* and self-fertile homomorphic relatives, we found no evidence of any consistent non-reciprocity of this type (Arnold & Richards, 1998).

In heterostylous genera such as *Primula*, the diallelic incompatibility system is essentially sporophytic in operation, and in multiallelic sporophytic self-incompatibility (SSI) systems, too, it seems that the self-incompatibility system is not implicated in interspecific incompatibility. When crosses are made between genetically modified oilseed rape (*B. napus*) and wild radish (*R. raphanistrum*), variation was detected both in the ability of oilseed rape mothers to accept radish pollen, and between radish fathers in their ability to sire hybrid embryos (Gueritain *et al.*, 2003).

It is possible that GSI self-incompatibility systems are sometimes, or in part, implicated in interspecific incompatibility, but in dioecious, heteromorphic and SSI plants at least, a completely different mechanism appears to mediate the failure of non-conspecific pollen to effect egg fertilisation. As yet, this mechanism has not been investigated in detail in any plant, and it is not clear whether heritable variation in interspecific incompatibility occurs between individuals of a parental species. In the latter case, one would expect relatively cross-incompatible variants to be selected through the Wallace Effect.

4.2.5 *Post-zygotic isolation*

More often than not, investigations after interspecific pollination reveal that several mutually reinforcing mechanisms affect fertility. For instance, when crosses were made between the sympatric species *Penstemon spectabilis* and *Penstemon centranthifolius* (which also normally differ in their pollinators), it was found that failure of pollen germination, pollen tube growth, fruit set and seed-set all contributed to reproductive isolation of the two species, although the importance of each reproductive stage in the overall fertility differed between reciprocal crosses (Chari &

Wilson, 2001). The basis of reproductive failure has a different explanation at each stage as is explored in the following sections.

4.2.5.1 *Embryo–endosperm balance seed incompatibility*

After interspecific fertilisation has occurred, seeds commonly fail to develop properly. Often this seed incompatibility is non-reciprocal, and so the fertile seed results from the cross in one direction, but fails when the cross is made the other way. For instance, when the reciprocal intergeneric crosses were made between self-compatible *Tellima grandiflora* and *Tolmeia menziesii* with late-acting self-incompatibility, pollen tubes grew normally in both crosses, but fertile seed was only set when *Tellima* was mother to the cross (Weiblen & Brehm, 1996).

The only mechanism mediating seed incompatibility for which we currently have any comprehension concerns endosperm–embryo balance. Our understanding of this little-studied phenomenon relies chiefly on the work of the co-workers of Valentine, the best examples of which are found in Woodell (1960a,b) and Valentine and Woodell (1963). In crosses between the primrose *Primula vulgaris*, cowslip *Primula veris* and oxlip *Primula elatior*, seeds failed after crosses were made, apparently because either the endosperm grew too slowly and the embryo starved (type A seed, which is small), or because the endosperm grew quickly and outcompeted the embryo (type B seed, which is bigger but often lacks an embryo). It was suggested that these imbalances resulted from the difference in genomic dose between the embryo, which receives one genome from each parent, and the endosperm, which receives two genomes from its mother, but only one from its father. It was noted that seed matured at intrinsically different rates among the three *Primula* species, with the oxlip maturing the slowest and the cowslip the quickest. (In the original papers, these were given genetic values, which in modern parlance appear to be the products of seed development rates and gene dosages). Interestingly, crosses made between the two species with the most different genetic values (oxlip and cowslip) were the only ones in which the seed development normally failed completely. Each species apparently brought into the hybrid seed its own intrinsic rate of maturation, and gene dosage conferred a particular speed of development to the independently formed but interdependent embryo and its endosperm nurse tissue (see Table 4.3).

One way of testing this model is to change the balance in the gene dose between the embryo and the endosperm using an artificially derived autopolyploid parent in the interspecific cross. When diploid cowslips were crossed with pollen from tetraploid oxlips, much of the seed was large and viable. In this case, it seems that the imbalance between the triploid OOC embryo and the tetraploid OOC endosperm does not present so grave a problem as that when embryos are diploid OC and endosperms triploid OCC or OOC (Valentine & Woodell, 1963).

It seems likely that failures of related species with different ploidies to form viable hybrid offspring also arise from embryo–endosperm imbalance, although this phenomenon should not be confused with the more commonly encountered sterility of hybrids with unbalanced ploidies that result from crosses between parents with different ploidy levels. Bushell *et al.* (2003) showed that embryo–endosperm

Table 4.3 The relationship between parental contributions to the embryo and endosperm genomes, and the size and viability of interspecific hybrid seeds formed between Oxlip, Primrose and Cowslip

Mother	Father	Embryo genome	Endosperm genome	Seed size	Percentage with embryo	Seed fertility	Seed Interpretation
Oxlip O	Primrose P	OP	OOP	Large (B)	25	Poor	Embryo somewhat outgrows endosperm
Oxlip O	Cowslip C	OC	OOC	Large (B)	0	None	Embryo fatally outgrows endosperm
Primrose P	Oxlip O	OP	OPP	Small (A)	80	Good	Endosperm somewhat outcompetes embryo
Primrose P	Cowslip C	PC	PPC	Large (B)	1	None	Embryo fatally outgrows endosperm
Cowslip C	Oxlip O	OC	OCC	Small (A)	0–30	None	Endosperm fatally outcompetes embryo
Cowslip C	Primrose P	PC	PCC	Small (A)	80–100	Good	Endosperm somewhat outgrows embryo

imbalance caused sterility when diploid *Arabidopsis thaliana* was crossed with pollen from tetraploid *Arabidopsis arenosa*. When an autotetraploid *A. thaliana* mother was used instead, however, the embryo–endosperm genome balance was restored and fertile hybrid seeds resulted.

As long as such models are applied to interspecific crosses where ecospecies have evolved intrinsically different reproductive attributes that we can assume are under genetic control, it is reasonable to assume that the male and female contributions to the embryo and endosperm do not differ in their mode of expression. Moreover, there is no reason to suggest that the male and female contributions to offspring tissues are differentially imprinted.

4.2.5.2 Genomic imprinting

Woodell and Valentine (1961) also tested their model using reciprocal intraspecific crosses between diploids and artificial autotetraploids. As crosses were made within species, there were no innate specific differences in seed development involved in these experiments. Nevertheless, they found that differences in gene dosage alone resulted in type A or type B seeds as predicted by their model of genetic values. Thus, if the male parent to a cross was tetraploid, the endosperm/embryo genome ratio was 1.33, but if the female parent was tetraploid the ratio would have been 1.67 (1.5 if both parents were diploid). As predicted, in the first case the seeds were small (type A) and in the latter case much larger but usually lacking an embryo (type B). In these intraspecific crosses there should be no genetic differences between the male and female contributions to the endosperm and embryo, yet the ratio of male-to-female contributions to these tissues was nevertheless still important. In more recent times such findings have received a molecular explanation in terms of genomic imprinting (Haig & Westoby, 1991). Gutierrez-Marcos *et al.* (2003) have recently suggested that such imprinting (the switching on or off of genes in offspring tissue depending on whether they have a paternal or maternal origin) may

have played a central role in genetic isolation and speciation in flowering plants, through interspecific seed incompatibility of the primula type. They even suggested that the evolutionary success of double fertilisation of the embryo and endosperm, which is unique to the angiosperms, might be explained in this way (Vinkenoog *et al.*, 2003). However, Eaton (1973) had already queried Valentine's view that seed incompatibility had a selectionist, Wallacean function, pointing out that innate differences in seed development that isolated the three *Primula* species through seed incompatibility had originally adapted these species to their ecological niches, and so isolation by seed incompatibility could be viewed as an accidental side effect of that adaptation. Three decades later, philosophical differences persist as to whether genetic isolation by genomic imprinting is central to the process of speciation, or an accidental by-product of it.

4.2.5.3 Seed sterility (F_1 embryo inviability)

Seed incompatibility results from paternal/maternal incongruities expressed in interdependent offspring tissues, the embryo and endosperm. However, other types of seed failure occur after interspecific crosses that can be attributed to intracellular incongruities between paternal and maternal material.

It is commonly observed that embryos and seeds resulting from interspecific crosses often fail to develop normally, and subsequently abort. Without a detailed embryological investigation, it is not always easy to distinguish this type of seed failure from seed incompatibility. One striking feature of seed sterility after interspecific fertilisation is its non-reciprocity. It is usually very difficult to predict the outcome of an interspecific cross made in one direction from the results of the reciprocal cross. Such results have been known for millenia. For instance, as described by Virgil, the viability and fertility of hybrids between the horse and the donkey are non-reciprocal. However, such relationships have perhaps never been so systematically investigated as by Michaelis (1954), who reported on the results of a large series of reciprocal crosses and backcrosses between some 10 species of annual *Epilobium*. Seed sizes, embryo sets, germinabilities, seedling vigour, physiology and fertility all usually varied between reciprocal crosses. Michaelis also reported a tendency for irregularities to become more pronounced in backcrosses from the F_1 to the original male parental species rather than those made to the female parental species. Damboldt (1965) obtained similar results for crosses between two familiar rock garden campanulas, *Campanula garganica* and *Campanula porcharskyana*. If the former parent was mother to the cross, seedlings were weak and chlorotic (although fertile if they survived), whereas the reciprocal hybrids were vigorous. *Penstemon* provides yet another example of non-reciprocal hybrid seed fertility (Chari & Wilson, 2001).

Michaelis explained such results by reference to the performance of genes of male parental origin in alien cytoplasm. His work preceded our understanding of the role and function of the DNA either in the nucleus or within organelles (his cytoplasm). Today, we know that a substantial component of the DNA occurs in circular form in the chloroplast and mitochondria, and is generally inherited maternally in flowering plants (but in the gymnosperms, extraordinarily, paternally). We know

that a number of key metabolic enzymes are of dimeric construction, part being coded by the nuclear DNA, and part by chloroplast DNA. Furthermore, organelles such as ribosomes, which are vital to the transcription and translation of the DNA, are inherited maternally.

Regrettably, there seems to have been no extensive study of interspecific hybridisation in the molecular era to match that of Michaelis, and a molecular investigation into the basis of hybrid seed inviability is seriously overdue. The days when experimental taxonomists made intensive crossing programmes in their investigations of the nature of the species are from a bygone age, and even then their complex crossing diagrams as seen for *Nigella* (Strid, 1970), *Elymus* (Snyder, 1950) or *Mimulus* (Vickery, 1964) typically concentrated on the performance, meiotic behaviour and fertility of the F_1 , and overlooked the processes that govern seed fertility and seedling viability.

Functionally, it is probable that there are no important differences in mechanism between hybrid embryos that form but abort, those that form but fail to germinate and those that germinate but fail to grow strongly enough to flower, such as the hybrid between the two common British poppies *Papaver rhoeas* and *Papaver dubium*, according to Stace (1989). In no case do we seem to understand exactly why hybrid embryos or seedlings fail, but the observation that such seedlings are frequently chlorotic, or even lack chlorophyll, suggests that maternally coded (chloroplast DNA) and paternally coded (nuclear DNA) components from different species may in these cases be insufficiently compatible to successfully form important metabolic enzymes, particularly those that govern photosystem II. Such an explanation might account for the observation that nuclear-cytoplasmic embryo inviability is less severe when hexaploid AABBDD wheats are crossed to diploid *Aegilops* species than when tetraploid AABB wheats are used as the male parent (Maan, 1992). Presumably, the *scs D* genome-located gene is compatible with chloroplast DNA genes in the *Aegilops* parents.

4.2.6 F_1 hybrid sterility

As we have seen, interspecific hybrid embryos or seedlings may be so feeble that they fail to flower. Where it has been found necessary to induce flowering in such hybrids so that they can be used as parents within breeding programmes, various techniques of embryo rescue and culture involving artificial media and aseptic technique have been employed.

4.2.6.1 Invasive sterile hybrids

In the majority of cases, once F_1 hybrids are formed they have in fact been found to be very vigorous, often more vigorous than the parents. A number of serious weeds have arisen after the intentional or accidental influence of man has caused previously isolated species to come together and hybridise. *Fallopia x bohemia* (Hollingsworth & Bailey, 2000), *Symphytum x uplandicum*, *Circaea x intermedia* (Raven, 1963), *Mimulus x robertsii* and several mints such as *Mentha x piperita* are

all examples of widespread, vigorous hybrids in the British flora that are sterile, or nearly so, but have become locally invasive and have largely replaced one or both parents. Such hybrids may have a considerable ecological impact. It follows that a perennial transgenic hybrid of this kind, whilst sterile, could reproduce vegetatively to become widespread in the environment and cause considerable ecological perturbations. Many staple crops are not perennial, but soft fruit such as strawberries (*Fragaria x ananassa*), fruit trees or timber trees that sucker (*Prunus* spp., *Populus* spp.) and herbs such as mints (*Mentha* spp.) all have the potential to disperse any transgenes introduced into the crop vegetatively, possibly but not necessarily within hybrids. The potential presence of transgenes that confer traits of ecological advantage would clearly enhance the likelihood of such invasions. *Consequently, it would be bad practice to introduce into any perennial crop with the potential for vegetative reproduction any transgene that has the potential to influence plant fitness or survival.*

4.2.6.2 *The causes of hybrid sterility: chromosome homology*

Many pairs of species form hybrids that are partially or completely fertile. These hybrids have the potential to backcross with one or both of their parents, and so transgenes present in one species (for instance a crop) have the potential to enter the gene pool of the other (which may be a related wild species). The factors influencing this possibility form the subject of the last section of this chapter.

While fertile hybrids do occur, it is very commonly found that when viable, vigorous hybrids are formed they are nevertheless completely sterile. This sterility nearly always results from a lack of homology between the chromosomes of the parental species, and so pairing (synapsis) between chromosomes at meiosis is irregular. When the parental species have similar chromosome numbers, the chief consequence of poor chromosome pairing is the formation of unpaired chromosomes (univalents) through diplotene to anaphase I. Univalents tend not to orientate on the spindle at metaphase I, and so they do not usually separate in a regular fashion and consequently often fail to enter the daughter nuclei at telophase I. This loss of one or more chromosomes in the daughter nuclei after meiosis in a hybrid typically results in meiotic products that abort, leading, for example, to empty pollen grains as, for instance, was shown by Grant (1966b) in *Gilia* hybrids (Figure 4.1). Only when the hybrid is polyploid (or perhaps formed between ancient polyploids) is there sometimes enough gene duplication between chromosomes for nullosomic meiospores to be viable.

For more than 80 years, thousands of cases have been reported in the literature of partial or complete F₁ hybrid sterility being explained by a partial or complete lack of homology between comparable chromosomes in the hybrid, or a difference in chromosome base number. Chromosomes then fail to pair and subsequently to separate regularly. One of the most striking recent reports concerns the aquatic Indian paddy weeds *Coix lacryma-jobi* (adlay) ($2n = 20$) and *Coix gigantea* ($2n = 12$). In this case, most or all of the chromosomes have fused into larger units (Robertsonian fusion) in the latter species (although, if the centromeres are diffuse, they might have

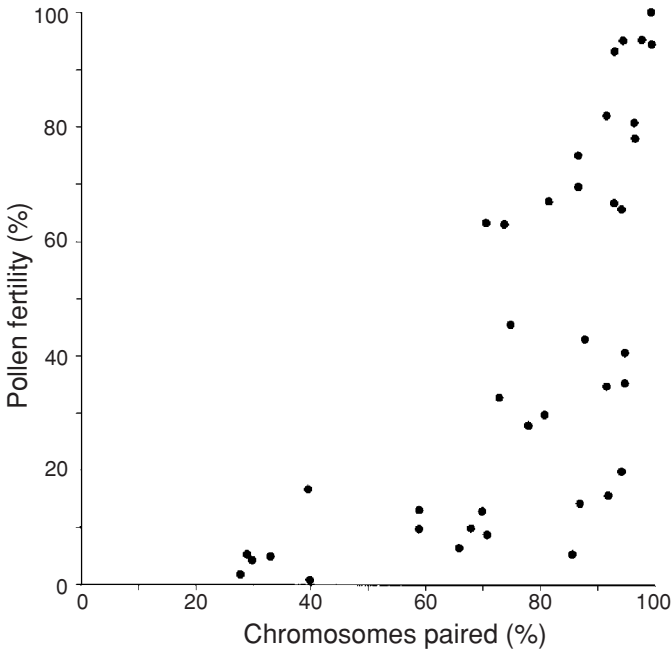


Figure 4.1 The proportion of chromosomes forming bivalents (x axis) and the percentage of fertile pollen grains in hybrids between *Gilia malior* and *Gilia modocensis* (Grant, 1966b).

split into smaller units to form the former species). In the hybrid meiosis ($2n = 16$), 10 small univalents and 6 large ones occur. Although the chromosomes appear to share a common origin, today there is no apparent homology between the genomes of these divergent species, and so the hybrid is totally sterile (Christopher *et al.*, 1997).

This is an extreme example. When related species form a hybrid, there are, broadly speaking, three possible classes of chromosome homology between the parental species (see Table 4.4). The third category, in which some homology remains between most comparable chromosomes in the two parents, is the most complex and the most liable to chance effects. The chance that the comparable chromosomes

Table 4.4 Chromosome pairing in interspecific hybrids

Chromosome pairing in hybrid	Consequences to hybrid fertility
Regular, all chromosomes homologous or nearly so	Regular disjunction, hybrid fully fertile
No chromosomes homologous, only univalents formed (as in <i>Coix</i>)	Most univalents do not disjoin, hybrid sterile
Some chromosomes homologous or partly so	Fertility low but some meiotic products viable

will form a regularly disjoining bivalent is related to the proportion of the parental chromosomes that are homologous. Theoretically, when some homology remains between all the pairs of comparable parental chromosomes, however slight, some meioses should form regular bivalents throughout, resulting in regular disjunction, and the formation of viable products with a full chromosome complement. How often this occurs will determine the fertility of the hybrid, and will be a function of the product of the shared homologies of all the chromosome pairs in the hybrid, but will be a very variable attribute (Grant, 1966b).

We still have a very imperfect appreciation of the molecular basis of chromosome homology and the abilities of comparable chromosomes, or sections of these chromosomes, to pair and subsequently to disjoin regularly at meiosis. Undoubtedly, the more gross changes that have occurred between comparable chromosomes (deletions, duplications, inversions, etc.), the less homologous they will be; the importance of such chromosomal mutations probably greatly exceeds the importance of DNA sequence order in determining homology. The role played by deletions in particular is highlighted by Morgan *et al.* (1986) who showed a strong relationship between bivalent formation and correspondence in DNA content for *Festuca* interspecific hybrids.

4.2.6.3 *The causes of hybrid sterility: irregular polyploidy*

Many genera display polyploid pillars, and so the base chromosome number of species occurs in multiples of the haploid count. Plants with two sets of chromosomes ($2x$) are called diploid, with four sets ($4x$) tetraploid and so on. Plants with more than two sets of chromosomes are called polyploid. Usually polyploidy has resulted from occasional failures of chromosome disjunction. When chromosomes occur in multiple sets (genomes) that derive from the same parent, and so are completely homologous, the polyploid is termed an autopolyploid. If the chromosome sets are derived from different parental taxa, and so the multiple sets vary between completely homologous to completely non-homologous, the polyploid is called an allopolyploid. When hybrids between parents of different ploidy have an uneven number of chromosomes, for instance three (triploid), they are usually completely sterile. This is perhaps to be expected, for three sets of genomes are not expected to disjoin regularly into two telophase I nuclei after meiosis I. Nevertheless, the causes of sterility are not always that simple. For instance, the vigorous and widespread hybrid watercress, *R. x sterilis*, between the diploid *Rorippa nasturtium-aquaticum* ($2n = 32$) and the tetraploid *Rorippa microphylla* ($2n = 64$) is triploid ($2n = 48$) and is, as the name suggests, completely sterile (Manton, 1950). Characteristically, for a cross between an allotetraploid and one of its diploid parents, the meiosis in the triploid hybrid normally forms 16 univalents and 16 bivalents (Figure 4.2). The genome derived from the diploid *R. nasturtium-aquaticum* in the triploid hybrid pairs with half the chromosomes derived from the tetraploid *R. microphylla*, while the other half of the *microphylla* chromosomes in the triploid are completely non-homologous with *nasturtium-aquaticum* chromosomes and so fail to find a partner and form univalents.

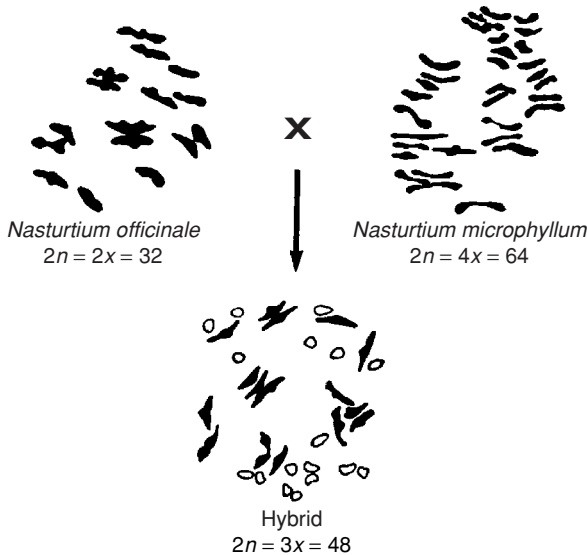


Figure 4.2 Meiotic chromosome spreads of cells at Metaphase I showing 16 bivalents in *Nasturtium officinale*, 32 bivalents in *Nasturtium microphyllum* but 16 bivalents and 16 univalents in the F_1 hybrid between the two.

Because a full diploid set of chromosomes in the triploid forms regular bivalents, it might be expected that it would disjoin regularly to form viable haploid meiotic products composed only of the *R. nasturtium-aquatica* genome. In practice, this seems rarely to happen in most triploid hybrids of this kind, perhaps because the bivalents rarely orientate regularly on the spindle in the presence of so many univalents. Very occasionally, *R. x sterilis* does set a few seeds, and it is assumed that the subsequent seedlings are diploid and resemble *R. nasturtium-aquatica*.

In the context of this chapter, the diploid *R. nasturtium-aquatica* (which is after all a crop) had been invested with a transgene, it is very unlikely that such genes could enter the genome of its allotetraploid wild relative *R. microphylla*. More relevantly perhaps, both the diploid crop and its triploid hybrid are so efficient at vegetative dispersal that transgenes advantageous to the crop would spread very effectively without sexual reproduction occurring at all. In common with all vegetative reproducers, therefore, watercress would form a most unsuitable subject for transformation with any transgene that confers advantage.

When diploids and their allopolyploid derivative cross, it is more usual to find that some homology exists between the two genomes in the allotetraploid, and so multivalent formation occurs in the triploid hybrid. In such cases, both the genomes in the triploid encounter severe problems when it comes to disjunction at meiosis. The minority genome still forms non-disjoining univalents, but some also form trivalents and quadrivalents with the other genome, and so both contribute to highly

unbalanced and usually non-viable meiotic products. The extent to which intergenomic multivalent associations are formed will depend not only on the degree of homology comparable chromosomes between genomes share, but also on the innate rate of chiasma formation at meiosis in the hybrid. Although such hybrids are normally very sterile, they are often not completely sterile; just occasionally the chance distribution of chiasmata, chromosome associations and disjunctions cause the occasional meiotic product to be balanced, usually as a diploid spore from a triploid. Backcrossing, if it occurs, will normally be to the tetraploid parent (Abbott *et al.*, 1992).

The most interesting consequence when fertile products result from intergenomic multivalent formation is the intergenomic recombination that results. As discussed in the final section of this chapter, even very rare hybridisation events can cause introgression, and so transgenes introduced into a crop can in theory become incorporated into the genome of a wild species of different ploidy.

However, hybrids apparently of unbalanced ploidy are not always highly sterile. For instance, triploid ($2n = 60$) hybrids between diploid *Dactylorhiza fuchsii* ($2n = 40$) and tetraploid *Dactylorhiza purpurella* ($2n = 80$) are quite fertile and regularly backcross to both parents, but especially to *D. purpurella*, giving rise to aneuploids, which themselves are quite fertile (Lord & Richards, 1977). The explanation that is sometimes given to this phenomenon suggests that although $x = 20$ is the lowest count now known in the genus, lower counts of $x = 10$ or even $x = 5$ might have occurred amongst its progenitors, and so diploid *D. fuchsii* is in reality an ancient polyploid in which chromosomes are already polysomic. As a consequence, apparently unbalanced genomes are less irregular than they first seem, being buffered by internal duplication. Such an explanation cannot however apply to *Euphrasia*, another example of a genus in which triploid hybrids are sufficiently fertile to backcross, particularly to the diploid parent. Here, the diploids only have $x = 11$, so they are unlikely to carry much internal polysomy. Nevertheless, 'incipient speciation occurs most strikingly between diploids and tetraploids. Recognised cases of this are so far all diploid: genes are presumed to pass into the diploid from a tetraploid and modify it in such a way that it comes to occupy a distinct habitat' (Yeo, 1975).

When species of different ploidy form a genomically balanced hybrid, the consequences are rather different. For instance, when a diploid crosses with a hexaploid that contains two of its genomes, the product is a tetraploid, and so disjunction can in theory occur evenly, with two genomes entering each of the daughter nuclei. If there is a good deal of homology between the diploid and at least one of the hexaploid genomes, and a fairly high rate of chiasma formation, the tetraploid hybrid may indeed prove to be moderately fertile. However, as the hexaploid genomes are present three times in the hybrid, low homology between the hexaploid and the diploid genome will greatly increase hybrid sterility.

Therefore, it is normally safe to conclude that parental species of different ploidies that hybridise to form a genomically unbalanced (for instance, triploid) hybrid are genetically isolated, because the hybrid is usually sterile. However, if one parent species is a crop species into which transgenes have been introduced, it should

be noted that the hybrid, although sterile, might nevertheless become an aggressive transgenic invader. Furthermore, triploid hybrids are rarely completely sterile, and so introgression of either the diploid or the polyploid genome from the other parent can occur. Consequently, transgenes could become incorporated into wild genomes with different levels of ploidy from the crop. In some cases, triploid hybrids are sometimes surprisingly quite fertile and these do not always have high base chromosome numbers.

4.3 Hybridisation, introgression and consequences of transgene spread

Most of this chapter has been devoted to mechanisms that isolate species, restricting the spread of genetic material such as transgenes from one species, perhaps a crop, into another co-occurring wild relative. It is clear that there are many often mutually reinforcing stages in the reproductive cycle at which genetic isolation can occur. It is equally true that any one, or all, of these mechanisms can be 'leaky' or can fail, and hence hybridisation can occur. *A striking feature of hybridisation is its unpredictability. If one, or even many, experimental tests for hybridisation between two species fail, it is still unsafe to conclude that hybridisation between these species can never happen. It is typical for hybridisation events to be rare.*

4.3.1 Hybrid swarms

Remarkably, there are some plant genera in which the creation of fertile hybrids to form so-called 'hybrid swarms' seems to be the rule rather than the exception. In wind-pollinated trees with good seed dispersal by wind (*Salix*, *Populus*, *Betula*) or animals (*Quercus*), hybridisation is commonplace, and so it is often considered that pure-bred populations are rarely found. Modern, molecular-based studies have been used to confirm these suspicions. A comparison of DNA sequence relationships between maternally inherited chloroplast genes and biparentally inherited nuclear genes has been instructive, especially in *Quercus*, as they provide very different types of evidence. Chloroplast genes have suggested that speciation in isolation occurred in 'overwintering' refuges during glacial epochs (Muir *et al.*, 2000). During re-immigration, long-distance colonisation by single acorns into virgin post-glacial territory can be tracked as these founding 'Eves' set up their local forest dynasties that today still carry the founder's chloroplast DNA. When the demes that had originally speciated during glacial maxima met again during their repeated journeys northwards, biparentally inherited genes have shown evidence of widespread hybridisation, and so species identity in, for instance, the oaks *Q. robur* and *Q. petraea* has become greatly confused (Bacilieri *et al.*, 1996).

Such widespread hybridity seems to characterise this genus. For instance, in southern Europe when *Q. pubescens*, *Q. frainetto* and other species of so-called 'white oak' were examined together with *Q. robur* and *Q. petraea*, remarkably

few basic chloroplast DNA haplotypes occurred in total, suggesting that speciation was fairly recent and its effects partly obscured by later post-glacial hybridisation (Petit *et al.*, 2002). Oak hybrid swarms also occur in China, Mexico and elsewhere. Nevertheless, careful examination in Ireland suggests that hybridisation is rarer than first thought, although 10% of births are still hybrid (Kelleher *et al.*, 2004).

Hybridisation in *Salix* seems even more pervasive, for it involves more species than in *Quercus*. However, the long-term propagation and spread of hybrid clones that are suitable for artefacts as arcane as the manufacture of cricket bats has confused the issue (Meikle, 1984), and the number of species that actually hybridise regularly in the wild is much smaller than first appears (Meikle, 1992; Tennant, 2004).

If hybridisation between trees is rife (and it often occurs between crop trees such as apples, *Malus domestica*, and wild relatives such as crabs, *M. sylvestris*, or between culinary plums, *Prunus domestica*, and wild sloe, *Prunus spinosa*), most other examples of hybrid swarms have resulted from the activities of man. By bringing previously isolated allopatric species into contact man has encouraged plant hybridisation over the last 100 years to an extent that was previously quite unprecedented. The results have often been spectacular. Anderson (1949) was perhaps the first to draw attention to such mass hybridisation, which was uncommon until the advent of the motorcar and transcontinental railroads. Among the relatively new phenomena that he reported were the hybrid swarms that had developed on the vast railroad marshalling yards of the American Midwest. Together with the hobos of American legend, seeds were carried across that great continent by freight trains, to come into genetic contact for the first time in their evolutionary history. In the sunflowers (*Helianthus*), swarms involving up to eight different species were recorded, a myriad of new genetic combinations being thrown up annually to be sorted by evolution. Sequential sampling over a period of time showed that evolution was indeed taking place very rapidly, even for subsets of the same hybrid population (*Helianthus annuus* × *Helianthus bolanderi*) in genetic contact with one another (Stebbins & Daly, 1961). One of the striking features of the evolution of these hybrid swarms over only 12 years was the selection for fertility, and so certain hybrid types had become much more fertile in this short time. Similar changes occurred rapidly in hybridising populations of *Helianthus divaricatus* and *Helianthus microcephalus* (Heiser, 1979).

Such studies are, of course, of more than academic interest. In the years since these hybridisation events were first recorded, increased dietary emphasis on vegetable oils has caused sunflower, *H. annuus*, to become a massively important warm-temperate crop. In the United States at least, sunflower crops commonly occur alongside wild, weedy populations of the same species and regularly hybridise with them, to the extent that 42% of the offspring of wild sunflowers can be fathered by crop sunflower pollen (Linder *et al.*, 1998). These authors conclude that ‘transgenes in cultivated sunflowers should readily introgress into sympatric wild populations, and their fate will be determined primarily by their (i.e. the transgene’s) fitness effects on the wild plants’.

4.3.2 Hybrid habitats

Man not only brought together previously allopatric species but also created open unspecialised habitats with limited competition in which maladapted but vigorous hybrids could thrive. As seral development proceeded, many of the hybrid genotypes failed to compete with the developing vegetation and disappeared, leaving relatively few genotypes that often possessed novel genotypes unknown in the populations prior to hybridisation (Arnold, 1992; Rieseberg, 1995).

The habitats that man created are often themselves novel and so present ideal opportunities for hybridisation. For many years, my students have studied a hybrid population of *Primula* on the embankments of a Northumberland (UK) reservoir constructed in 1840. The primrose, *P. vulgaris*, is essentially a spring-flowering, woodland plant that thrives in partially shaded sites with fertile brown-earth soils that remain moist but moderately well drained, at least in spring. The cowslip, *P. veris*, is a plant of open sites in full sun and grows on soils that are well drained, typically infertile and rather shallow. The former species thrives in north-facing sites at the bottom of the embankments, close to the surrounding ditch, whereas cowslips occur exclusively on the opposing south-facing embankments, high up, in dry sunny positions. Although F₁ hybrids can only occur when cowslips are the female parent to the cross (see Table 4.3, p. 92), hybrids occur quite commonly (Figure 4.3) but mostly halfway up the reservoir embankment, in an intermediate habitat where neither parent is particularly common. The F₁ plants are fairly fertile and backcross hybrids are commonly encountered, but most if not all have backcrossed to the cowslip, and not to the primrose. Here is a good example of a hybrid plant occupying a hybrid habitat.

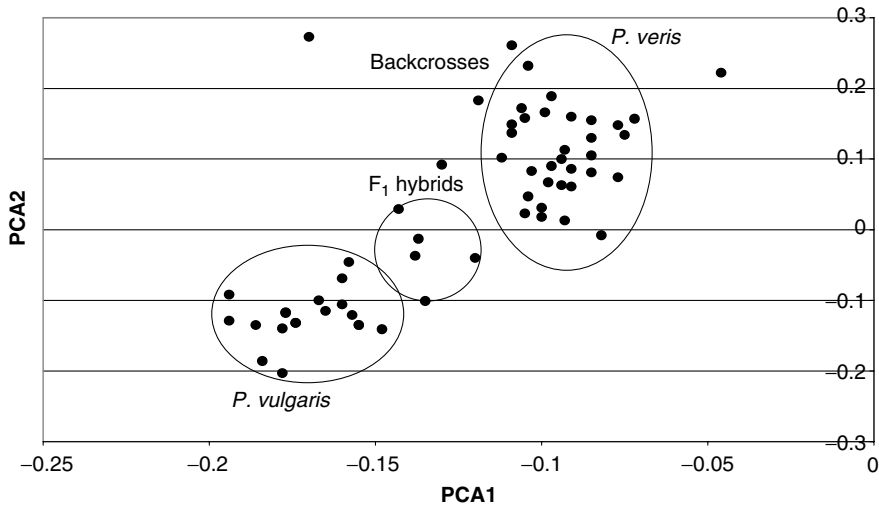


Figure 4.3 Principle components analysis of a hybrid swarm between *Primula vulgaris* and *Primula veris* in a Northumberland reservoir.

One of the earliest examples of hybrid population analysis also provides a good example of hybrid habitats. *Iris fulva* is typical of damp shady woodlands in the American South, whereas *Iris hexagona* var. *giganticaerulea* is a marsh plant of the Mississippi Delta, growing in full sun, which thrived in areas prone to the periodic floods that used to inundate the flood plain of that great river. Riley (1938) examined the populations colonising the engineering schemes that were attempting to reclaim the flood lands as pasture and cotton fields. Each of the reclaimed fields was surrounded by a dam (levee) with an accompanying ditch to drain away surplus water. The levees had allowed the shade-loving, flood-intolerant *I. fulva* to penetrate well into the Mississippi marshlands where it was able to hybridise with *Iris hexagona*. When flood damage caused the levees to collapse and they were abandoned, a variety of stabilised hybrids colonised the intermediate habitats that resulted, neither the levees nor the water meadows.

4.3.3 Introgression

The term 'introgression' or 'introgressive hybridisation' was first coined by Anderson (1949) to describe a situation where two species formed occasional, mostly sterile hybrids, but the F₁ hybrid was sufficiently fertile for occasional backcrosses to occur, usually in the direction of one parent only. Backcrosses were more fertile than their hybrid parent, and so they backcrossed again chiefly to the species parent. The net result tended to be that one of the parents became more variable, having acquired genes from the other parent, and on occasion this may have allowed the introgressed parent to evolve into a new niche.

Over the intervening years, Anderson's strict definition of introgression became somewhat diluted as it came to be used to describe conditions closer to the stabilisation of hybrid segregates from a hybrid swarm (for which Anderson himself was partly to blame). However, there are some good examples of genuine Andersonian introgression in the literature, and modern molecular techniques have confirmed some previously suspect examples. For instance, not long after the introduced Oxford Ragwort *Senecio squalidus* became established around Britain in the nineteenth century, it was noticed that in its company grew groundsels *Senecio vulgaris*, which like Oxford Ragwort possessed ray florets, promoting outcrossing (the mostly selfed groundsel normally has no ray florets). The suggestion that this potentially advantageous feature might have arisen from hybridisation and introgression was doubted at first, because when the triploid hybrid between the two species was synthesised, it proved to be highly sterile. Nevertheless, Abbott *et al.* (1992) were able to use evidence from isozymes to show that ray florets in groundsel had indeed arisen as a result of introgression from Oxford Ragwort, and that such introgression episodes had been polytopic. Rayed groundsels from York, for instance, have a different origin from those in Edinburgh.

Modern cytological methods (especially so-called 'chromosome painting' by genomic in situ hybridisation) have demonstrated that introgression is perhaps more common than was first thought. Introgression takes place at the level of the

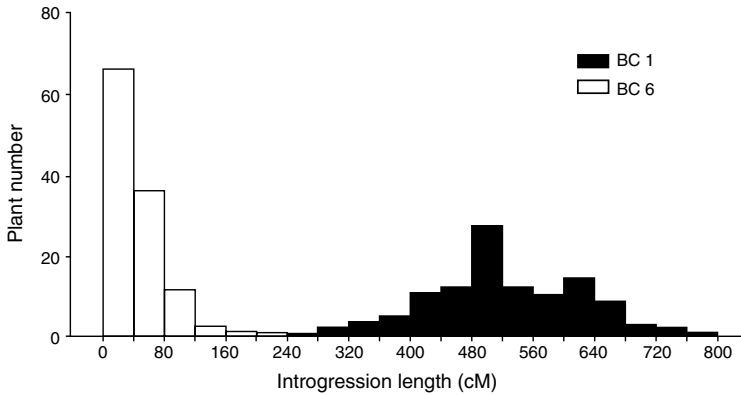


Figure 4.4 A comparison of the size of 'foreign' chromosomal inserts from *Lycopersicon pennellii* in the genome of the tomato *Lycopersicon esculentum* between the first backcross generation (black histograms) and after six generations of repeated backcrosses. Recombination between the genomes fractionates the 'foreign' inserts (Eshed *et al.*, 1992).

chromosome. High levels of sterility in the F_1 may well be caused by a low level of homology between the chromosomes originating from the two parents involved. However, homologous regions of comparable homeologous chromosomes that do pair will exchange segments, and so chromosomes that enter the backcross from the F_1 will show a greater level of homology to those originating from the species parent, with the consequence that the backcross will be more fertile than the F_1 . Through repeated recombination and backcrossing, smaller and smaller, and fewer and fewer segments of foreign chromosome segments will be retained in the introgressed species (Eshed *et al.*, 1992). This is clearly demonstrated in Figure 4.4.

This is a subject that deserves close attention in the context of the present volume. Introgressed chromosome segments are most likely to survive within the gene pool of backcross segregates if they contain genes promoting exceptional fitness, such as some transgenes that might have been transferred from a GM crop to a wild relative (Linder *et al.*, 1998).

A classic example of introgression was provided by Woodell (1969). In the United Kingdom, the primrose is common and widespread, whereas the oxlip *P. elatior* is a rare and localised plant. Here on the edge of its considerable range the oxlip is untypically confined to dense, swampy fen carr in East Anglia, a habitat that the primrose was formerly unable to penetrate, and so the two species remained completely isolated (Figure 4.5). However, a combination of drainage and the coppicing of the willow and alder in fen carr had latterly allowed the primrose to invade oxlip territory. When the species come into contact, hybrids occur fairly commonly, mostly in the first instance through crossing to a primrose mother (see p. 102). Hybrids are moderately fertile, and so in the years that followed coppicing, hybrid swarms resulted (Figure 4.6). However, if the coppice was abandoned, populations reverted to nearly pure oxlips in less than 50 years, but with definite signs of introgression by

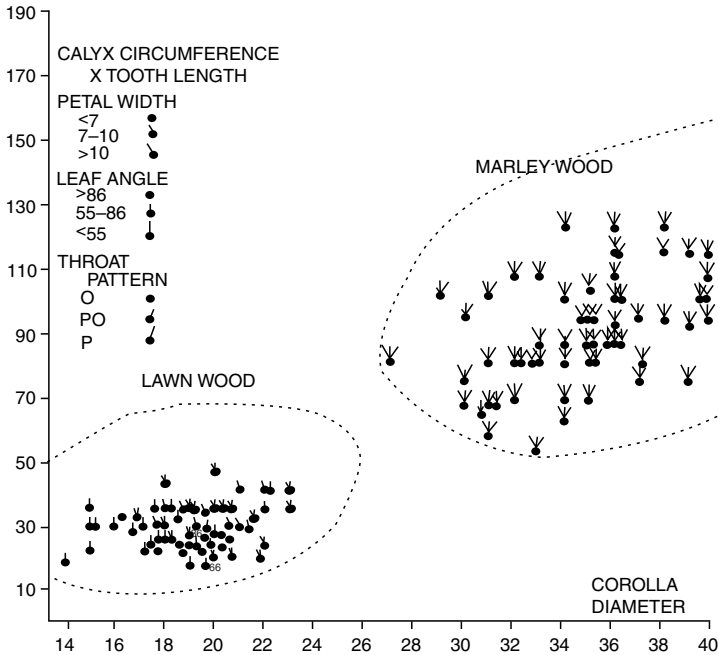


Figure 4.5 Scattergram based on two continuous characters and three tristate characters showing complete discrimination between pure populations of the oxlip (Lawn Wood) and primrose (Marley Wood).

primrose genes (Figure 4.7). More worrying perhaps was the fate of populations with continued coppice management. Former oxlip populations had reverted to stands of nearly pure primroses, although these too had some indication of introgression, this time with oxlip genes (Figure 4.8).

4.3.4 Hybridisation and extinction

Woodell's work introduces a further topic that is relevant to the possible movement of transgenes through hybridisation. In many former oxlip populations, pure oxlips have now become very rare, and hence we can suggest that oxlips are being hybridised out of existence by primroses. This is not an isolated example, even in the United Kingdom. Partial drainage of fenland has resulted in the virtual extinction of pure populations of the fen violet *Viola persicifolia* in many sites where most or all such plants are now hybrids with *Viola canina*, and nearly all the (rather transient) British populations of the rare lake-shore *Ranunculus reptans* rapidly lost their identity a few years after discovery through hybridisation with lesser spearwort *Ranunculus flammula*.

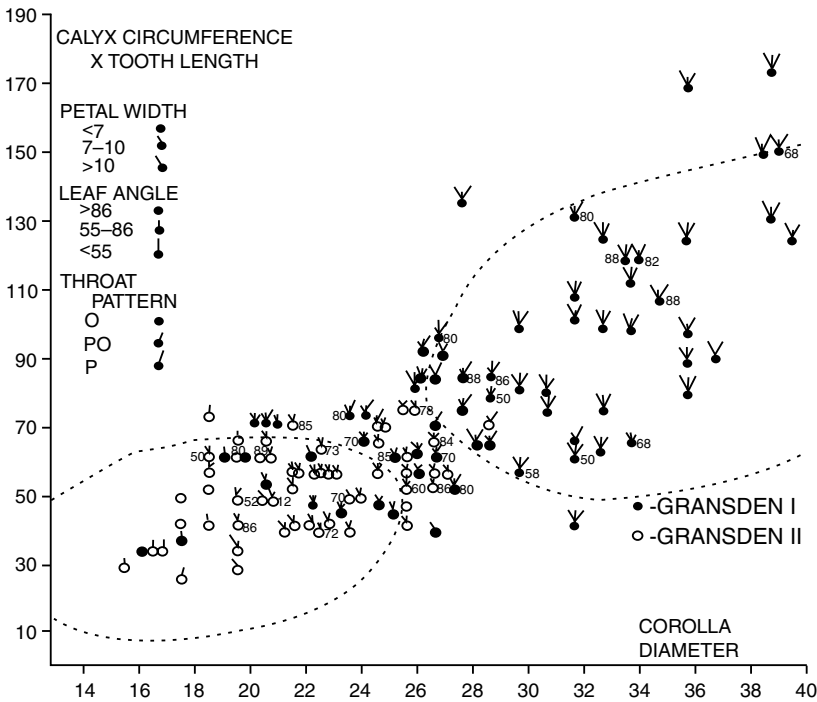


Figure 4.6 Scattergram of hybrid swarm between oxlip and primrose, using the same discriminants as in Figure 4.5. The limits of pure species populations are shown by the dotted lines.

Clearly the potential exists for an aggressive, GM crop to go native, and hybridise with a related wild species, to the detriment of the latter. One would expect hybrids that contain a fitness-promoting transgene to outcompete the wild species, to the detriment of the latter. The demise of the native British cordgrass *Spartina maritima* in the face of competition by its much more vigorous hybrid offspring *Spartina x townsendii* and the allopolyploid *Spartina anglica* is an illustration of what might happen (although in those cases of course, no transgenes were involved). In this respect, it is important to recognise that the key issue is whether the GM crop presents a significantly greater risk than the non-GM equivalents. For this, cognisance should be taken of the number and position of loci involved in conferring fitness advantage. In the examples given above, it intuitively seems likely that the effects of many interacting loci will be involved in causing the observed changes to phenotype. One might argue, therefore, that a single transgene poses only a slightly greater threat than exists already and so will tend to exacerbate an existing problem rather than create new problems. This is because only one aspect of phenotype will probably be affected by the transgene itself and other regions of the introgressed genome will be no different to those from non-GM crops, apart from the enhanced transmission of gene linked to the transgene (a process known as gene hitchhiking). However,

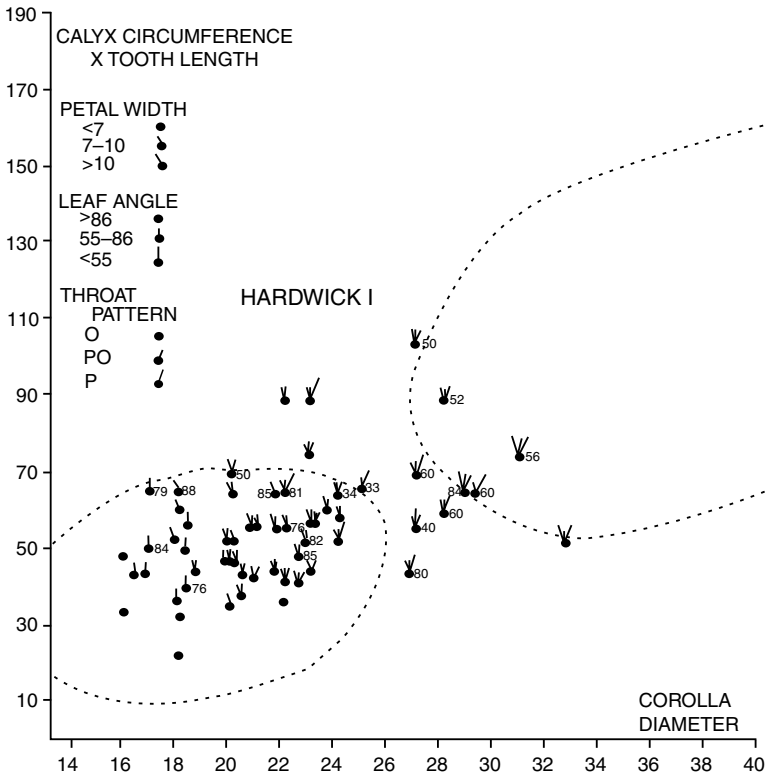


Figure 4.7 Abandoned coppice: introgressed oxlip population, using the same discriminants as in Figures 4.5 and 4.6 and showing the limits of pure species populations (dotted lines).

there is a growing trend for new GM lines to contain several transgenes, a process known as transgene stacking. It is entirely plausible that several transgenes in a stacked GM crop could confer greatly enhanced fitness in the recipient species. In this instance, therefore, the GM crop would pose a much greater risk of extinction by hybridisation than the non-GM equivalent. As always then, the key factor lies in the identity and function of the transgenes.

4.4 Concluding remarks

Initial releases of transgenes into the environment will always happen within populations of crop plants, and the greatest potential for transgenes to transfer from crop fields into the wider environment is provided by the crop plants themselves. Most of the wild relatives of crop plants with which transgenic crops could potentially hybridise are themselves weedy species, and so transgenes are much less likely to enter plant populations typical of stable or climax communities.

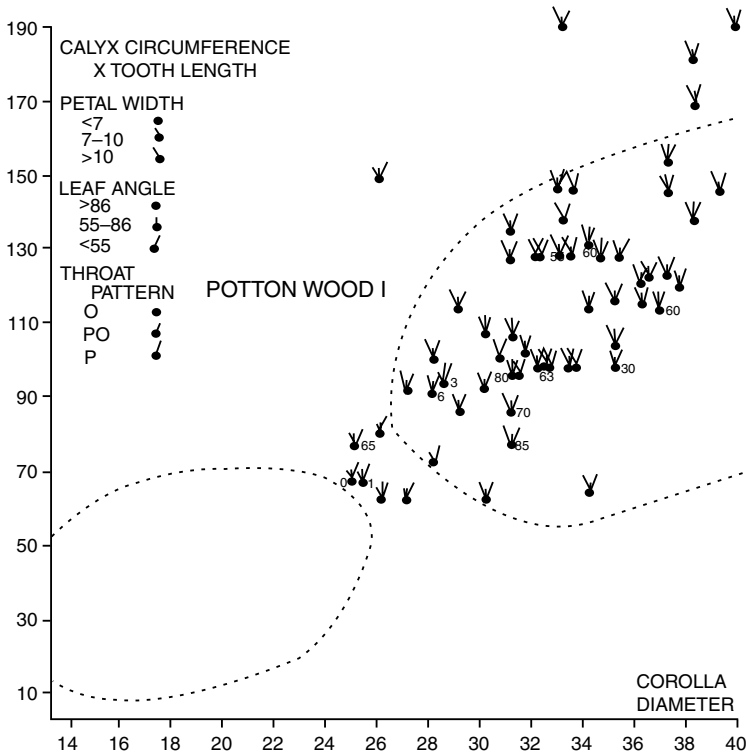


Figure 4.8 Continued coppice: introgressed primrose population, using the same discriminants as in Figures 4.5 and 4.6 and showing the limits of pure species populations (dotted lines).

Nevertheless, hybridisation is a pervasive feature of plant populations, and a striking feature of most examples of plant hybridisation is their rarity. A quantitative approach to the likelihood of hybridisation and other measures of exposure (see Chapter 7) is pointless unless new gene technologies or management procedures can dramatically change the situation beyond the scope of previous experience. The lessons we learn from examples of allopolyploid speciation, introgression, interspecific incompatibility, seed incompatibility and interspecific seed sterility are that major evolutionary developments can result from single, unpredictable and extremely rare hybridisation events.

Neither is the supposed sterility of a hybrid plant particularly important in itself. A sterile transgenic hybrid perennial can, if survival is favoured by the transgene, nevertheless prove to be an extremely aggressive invader, as many sterile hybrids have already demonstrated. Furthermore, annual GM hybrids showing extremely low levels of fertility can also pass their transgenes into wild relatives following rare backcrossing events, since thereafter fertility will gradually rise.

It is important that attempts are made to predict what environmental damage might result from the release of a transgenic crop away from the crop system. Perhaps the

most important attributes to be examined are the increased levels of fitness that possession of the transgene confers in the general environment. For instance, if a transgene confers resistance to a herbicide, what is the relative fitness of such transgenics to wild-type genotypes in the absence of the herbicide? If the transgene confers protection against damage from certain classes of invertebrate herbivore, how much of an advantage is this away from the crop system? It is these potential advantages that might create an environmentally damaging GM plant, either of the crop itself or a relative. Hybridisation between most GM crop species and their wild relatives is, sooner or later, a certainty without extreme risk management procedures. The critical question is will there be any ecological consequences arising from hybridisation?

Behaviour of transgenic plants away from the agronomic environment is, at best, extremely unpredictable. In the absence of the herbicide, triazine-resistant *B. rapa* is outcompeted by wild strains in all environments tested. For fathen, *Chenopodium album*, exactly the opposite result was found. In every environment tested, triazine-resistant genotypes outcompeted the wild types, and proved to be extremely aggressive in the absence of the herbicide (Plowman *et al.*, 1999). It is also difficult to be sure that any other type of transgenic phenotype would be selectively neutral. To take one example, a number of strains of tomato, *Lycopersicon esculentum*, now have enhanced levels of ascorbic acid (vitamin C) after genetic modification. This modification, which has been engineered for the sake of human health, might be thought to have few effects on plant fitness, but it is now recognised that the main novel attribute that allows some plants to evolve resistance to high levels of ozone is raised levels of ascorbic acid (Zheng *et al.*, 2000). In some ozone-rich environments, it is conceivable that only tomato fruits engineered to produce abundant vitamin C might survive, causing these genotypes to predominate.

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5 Rare hybrids and methods for their detection

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5.1 Introduction

Risk assessment of a GM crop requires knowledge about the extent of gene flow between the modified crop and the recipient species, as well as about survival of the resulting progeny. The following general risk algorithm can describe the consequences of gene flow: Risk = exposure \times hazard (Mackenzie & Henry, 1990).

The exposure component of the equation essentially comprises a series of events governing the frequency of effective gene exchange between the GM crop and the recipient populations. This variable is multifaceted and includes consideration of the number of hybrids, hybrid fertility and fitness, linkage and gametic drag associated with genes from the crop genetic background (see Chapter 6), the extent of genetic interchange between genomes, the rate of gene flow between recipient populations and the demography of the recipient species and its ecology. These aspects have a natural sequential order and can be usefully modelled as a pathway or matrix of interconnected events, the first of which is hybrid formation itself (Wilkinson *et al.*, 2003). It is important to consider all components of the introgression pathway when attempting to assemble a comprehensive estimate of exposure related to gene flow, although the task is made difficult by the fact that many factors vary both in time and space. The first task is to establish whether hybrids are at all likely to form under natural conditions and if so, in what numbers. This undertaking is relatively simple in cases where hybrids are relatively numerous, but becomes increasingly onerous as hybrids become scarcer. This chapter therefore presents the current state of knowledge on occurrence of rare hybrids, and advocates methods and approaches that can be used for their detection. When successful fertilisation of a plant has been accomplished, and seeds, propagules or hybrid plants have been produced, easy methods are needed for detection of the hybrids and for quantification of hybrid occurrence (the term 'hybrids' is used in the broad sense covering F₁ hybrids and later generations of introgressed plants). The methods described cover both detection of transgene flow and flow of endogenous crop genes. In most of the studies of crop gene flow, the analysis has been based on verifying transfer of natural markers. There is no reason to believe that transgenes will be introgressed differently from endogenous genes and markers, except in exceptional cases such as when the transgene affects reproductive behavior. The rate of stable transgene recruitment and secondary spread to other recipient populations, however, may increase or decrease because of selection acting on the transgenic phenotype. In comparison, most anonymous markers and endogenous genes are assumed to be selectively neutral.

5.2 Gene flow – to what extent will it take place?

Gene flow has played and continues to play an important role in the evolution of plants (Rieseberg & Wendel, 1993; Arnold, 1997; Ellstrand, 2003). Indeed, 12 of the 13 most important cultivated crops have been shown to hybridise with at least one of their wild relatives somewhere within their distribution area (Ellstrand *et al.*, 1999). While in many cases, the ecological consequences have either been insignificant or cryptic, the consequences could be significant. In rice (*Oryza* sp.), for example, ecological consequences arising from the spontaneous crosses could threaten the endangered relative *O. rufipogon* ssp. *formosana* (Song *et al.*, 2003). In many of the most common crop genera, however, gene flow threatens rather to enhance the weediness of the already weedy recipients, providing them with increased adaptability to cultivated conditions and thereby cause significant agricultural problems. In these cases, the rate of gene flow need not be great for the effect to become manifest and widespread, provided that the selective advantage is sufficiently large and gene flow occurs between populations of recipients (allowing secondary spread). On the other hand, where only a subset of wild or weed population of crop relatives is exposed to the GM crop and populations are dispersed and genetic exchange between them is limited, the outcome of GM crop to wild relative gene flow is likely to be spatially restricted. Thus, different scenarios require different levels of gene flow in order to cause realisation of the unwanted environmental outcome (assessment endpoint, see Chapter 7). It is therefore important that we are able to effectively predict the location and extent of gene flow, between the crop itself and the wild relative, and also between populations of wild relatives. For this, we first need to understand the underlying mechanisms limiting gene exchange between crops and their relatives.

5.2.1 Barriers to gene flow

Clearly, hybridisation and subsequent introgression between cultivated and wild plants is not an unusual phenomenon. However, the magnitude of gene flow between plants is determined by a number of isolating mechanisms. Levin (1978) made a classification of these isolating mechanisms. The reproductive barriers to gene flow are described in more detail in Chapter 4, but some examples of factors that decrease or increase gene flow are given below:

- *The degree of overlap of distribution area and flowering period for crop and recipient.* Weedy relatives growing in close physical contact with the crop are especially prone to hybridisation, as for example when the relative is a weed in fields of the crop. This is the case for the crop oilseed rape, *Brassica napus*, and its relatives *Brassica rapa* and *Raphanus raphanistrum*.
- *Environmental factors such as the frequency and density of the two hybridising genotypes, the surrounding vegetation and the pollinators present.* For example, varying the proportions of genotypes in mixed populations of oilseed rape, *B. rapa* and their F₁ hybrids has a large effect on seed output (Hauser *et al.*, 2003).

- *The reproduction system in crop and wild species.* Generally, more hybrid plants can be found in perennial plant groups where outcrossing predominates and where clonal survival of hybrids is possible by vegetative propagation or agamospermy. Perennial rye grass (*Lolium perenne*) and poplar (*Populus* sp.) are good examples of such plants.
- *The cross compatibility between crop and wild relative.* As a rule, the closer that two taxa are related to each other, the more cross-compatible they are and so the more introgressive gene flow will occur. It therefore follows that intraspecific gene flow occurs more readily than interspecific gene flow, since the cross compatibility is high and improved offspring fitness (heterosis) is common. In the case of interspecific gene flow, genetic barriers may reduce the production of offspring and the offspring often suffer from a reduced fitness (see, for example, review by Arnold, 1997). It should also be noted that different genotypes of the same species may have different potential for hybridisation, a phenomenon observed in spontaneous crosses between oilseed rape and *Raphanus raphanistrum* (Baranger *et al.*, 1995; Gueritain *et al.*, 2003).
- *The fitness advantage provided by the transgene or crop genes linked to the transgene.* If the new gene is highly advantageous there will be a strong selection for the plants that receive the gene. Genes that increase plant productivity and provide resistance to stress factors such as drought, cold and plant pests may be selected and will then speed up the gene transfer process. Hall *et al.* (2000) described the stacking of several genes for herbicide tolerance in oilseed rape volunteers as a result of spontaneous intercrossing. Such multiresistant plants could be highly advantageous in agroecosystems in which herbicide application is the main means of weed/volunteer control. Transfer of a few extra genes that do not provide advantages to the recipient are unlikely to present a metabolic burden and thereby reduce the fitness of the plant (Snow *et al.*, 1999). As a transgene is not transferred alone but linked to other crop genes, the recipient plants may suffer or benefit from these linked crop genes. Thus the position of the transgene integration site within the host genome has importance in affecting the probability of successful introgression.

5.3 Plants have different potential for dispersal of genes

5.3.1 Species groups

Formation of F₁ hybrids is the first step in the gene transfer. Ellstrand *et al.* (1996) found hybridisation to be non-randomly distributed among taxa. They reviewed five floras, two from European areas (see Table 5.1), two from North America and one from the tropics. They found that spontaneous hybridisation is not ubiquitous among plant families and genera; in each of the examined floras, only 6–16% of the genera had hybrids reported. More than half of the hybrids were accounted for by 5–21% of the genera in which hybrids occurred. Generally speaking, the species groups

Table 5.1 The six families and four genera from the British Isles and Scandinavia with most hybrids¹

Flora	Families ²	Hybrids	Genera	Hybrids
British Isles	Scrophulariaceae (5)	88	<i>Euphrasia</i>	71
	Salicaceae (6)	55	<i>Salix</i>	55
	Rosaceae (3)	53	<i>Epilobium</i>	43
	Onagraceae (7)	46	<i>Rosa</i>	36
	Poaceae (2)	45		
	Asteraceae (1)	41		
Scandinavia	Cyperaceae (4)	30	<i>Carex</i>	25
	Poaceae (2)	25	<i>Salix</i>	15
	Asteraceae (1)	18	<i>Viola</i>	7
	Salicaceae (6)	15	<i>Calamagrostis</i>	5
	Rosaceae (3)	13		
	Dryopteridaceae (8)	9		

¹Modified from Ellstrand *et al.* (1996).

²Families are ranked after species number.

with the highest incidents of hybrids were outcrossing perennials with reproductive modes that stabilised hybridity, for example vegetative spread or agamospermy. It is recognised that the flora information could be biased according to special interests. However, when considering the possibility for gene flow, distribution of hybrids among taxa may give hints to the more likely 'hot-spot' genera.

Tentatively, crop species and their gene recipients can be divided into categories according to the potential environmental impact from gene flow. Highly competitive plants like perennial ryegrass (*Lolium perenne*) and poplar (*Populus* sp.), which are perennial, outbreeding and fast proliferating, have the potential to become even more competitive with new genes transferred (DiFazio *et al.*, 1999). Moreover, plants such as oilseed rape (*Brassica napus*), oat (*Avena sativa*) and beet (*Beta vulgaris*) outcross with related taxa but the hybrid offspring are less competitive and rarely survive in climax communities unless disturbances are introduced. Finally, annual or biannual plants that survive and reproduce only when cultivated, mainly self-pollinating and with no obvious cross-compatible recipients, are unlikely to be more competitive unless their fitness is strongly improved by a new gene. In Europe, sunflower (*Helianthus annuus*) is an example of such a crop; however, the sunflower scenario is somewhat different in the United States, as cross-compatible recipients are a concern (Burke *et al.*, 2002; Burke & Rieseberg, 2003). This highlights the importance of considering geographical context when attempting to measure hybridisation.

Evidence of introgression is far more difficult to demonstrate than hybridisation and many of the current references reporting probable introgression between crops and wild relatives rely on circumstantial evidence (Heiser, 1973; Doebley, 1990). Indeed, Rieseberg and Wendel (1993) suggested that natural introgression was documented in less than 70 cases. However, few species pairs with a potential for gene exchange have been investigated in any kind of detail. Heiser (1973) examined cases

Table 5.2 Some European crops with cross-compatible wild or weedy relatives

Crop species (donor)	Wild/weedy recipient
Sugar beet (<i>Beta vulgaris</i> ssp. <i>vulgaris</i>)	Sea beet (<i>B. vulgaris</i> ssp. <i>maritima</i>)
Poplar (<i>Populus</i> spp.)	Black poplar (<i>P. nigra</i>) and Aspen (<i>P. tremula</i>)
Lucerne (<i>Medicago sativa</i>)	Sickle medie (<i>M. falcata</i>)
Oilseed rape (<i>Brassica napus</i>)	Wild turnip (<i>B. rapa</i> (= <i>B. campestris</i>)), wild radish (<i>Raphanus raphanistrum</i>), hoary mustard (<i>Hirschfeldia incana</i>) and field mustard (<i>Sinapis arvensis</i>)
Radish (<i>Raphanus raphanistrum</i>)	Wild radish (same species)
Ryegrass (<i>Lolium perenne</i> and <i>Lolium multiflorum</i>)	Wild ryegrass (same species), <i>Festuca</i> species
White clover (<i>Trifolium repens</i>)	Wild white clover (same species)
Carrot (<i>Daucus carota</i> ssp. <i>sativus</i>)	Wild carrot (<i>D. carota</i> ssp. <i>carota</i>)

of possible introgression involving such crops as maize (see also Doebley, 1990), sorghum, rice, tomato and potato, and Wilson (1990) reviewed the evidence for introgression in squash. There are several suggestions in the literature as to which crop species can potentially transfer their transgenes to wild or weedy relatives via natural processes, but there are a precious few that provide any definitive genetic evidence in support of the claims. Table 5.2 lists some of the important cases relevant to European conditions (based on Doebley, 1990; Dale, 1992; Raybould & Gray, 1993; Jørgensen, 1999; Ellstrand *et al.*, 1999; Ellstrand, 2003). The central difficulty lies in establishing whether a rare marker or gene that is common in the crop and present only occasionally in the relative is genuinely evidence of introgression or simply representative of the natural background variation in the wild relative.

5.4 The hybridisation potential of major European crops

The potential for gene flow from the 10 most frequently cultivated crops in Europe is briefly described below, ranking the crops according to the size of the cultivated area. Knowledge about rare hybrid formation with these crops is mainly from North America. Table 5.3 summarises the information about crops, their reproductive systems and cross-compatible relatives.

5.4.1 Wheat, *Triticum aestivum* (17 300 000 ha)

The widely cultivated bread wheat (*Triticum aestivum*) cultivars are predominantly self-fertilising although landraces may exhibit some degree of outcrossing by wind pollination (Tsegaye, 1996). For those species/varieties in which outcrossing does

Table 5.3 The ten most common crops in Europe and their potential for gene flow

Crop	Distribution	Modes of reproduction	Means of dispersal	Wild cross-compatible relatives in Europe
Wheat (<i>Triticum aestivum</i>)	All European countries	Self-fertilisation, low frequency of outcrossing	Pollen (wind dispersal) and seeds	<i>Triticum</i> species (i.e. wild emmer <i>T. turgidum</i>), <i>Aegilops</i> species (i.e. <i>A. cylindrica</i>)
Barley (<i>Hordeum vulgare</i>)	All European countries	Self-fertilisation, low frequency of outcrossing	Pollen (wind) and seeds	<i>H. vulgare</i> ssp. <i>spontaneum</i>
Maize (<i>Zea mays</i>)	South Europe	Cross-fertilisation, low frequency of selfing	Pollen (wind) and seeds	None. (Pollen dispersal between fields very likely)
Pumpkins for fodder (<i>Cucurbita</i> sp.)	South Europe	Outcrossing	Pollen (insects) and seeds	None. (Cross-fertilisation between fields and varieties likely)
Olives (<i>Olea europaea</i>)	Mediterranean	Cross-fertilisation	Pollen (wind) and seeds	Wild olive (some are old clones of the crop)
Oilseed rape (<i>Brassica napus</i>)	North and mid Europe	Self-fertilisation, some cross-fertilisation (10–30% of seeds)	Pollen (insects and wind) and seeds	<i>B. rapa</i> , <i>B. oleracea</i> , <i>B. carinata</i> , <i>B. juncea</i> , <i>Raphanus raphanistrum</i> (<i>Sinapis arvensis</i> , <i>Hirschfeldia incana</i>). (Pollen dispersal between fields very likely)
Grapes (<i>Vitis vinifera</i>)	Mid and South Europe	Mainly self-fertilisation, some outcrossing	Pollen (insects) and seeds	<i>V. vinifera</i> ssp. <i>sylvestri</i>
Sunflower seed (<i>Helianthus annuus</i>)	South Europe	Cross-fertilisation	Pollen (insects) and seeds	None. (Pollen dispersal between fields very likely)
Sugar beet (<i>Beta vulgaris</i> ssp. <i>vulgaris</i>)	North and mid Europe	Cultivated types are harvested before bolting. Seed production from crosses between male sterile and pollen-producing lines	Pollen (wind and insects) and seeds	Wild and weedy beets (i.e. <i>B. vulgaris</i> ssp. <i>maritima</i>)
Oats (<i>Avena sativa</i>)	North Europe	Self-fertilisation, low frequency of outcrossing	Pollen (wind) and seeds	<i>A. fatua</i> , <i>A. sterilis</i>

occur, the large quantity of pollen generated by fields of the crop means that there is a potential for long-distance pollen flow, and rendering hybridisation between varieties likely (Hucl, 1996; Tsegaye, 1996), especially between varieties/species of similar ploidy level. While such observations suggest that hybrids will form, they have little value in predicting the extent of hybrid formation between agricultural fields. The need for this kind of information is becoming increasingly important and the issue of coexistence between GM and non-GM farming systems (particularly organic farming) require information on cross-fertilisation rates between neighbouring fields. However, recent field experiments (Matus-Cadiz *et al.*, 2004) provide the first data of reasonable scale on this issue and their results indicate low levels of gene flow between donor and recipient plots, with rates ranging from 0.5% at the shared boundary and falling to 0.005% at 300 m. There is only one wild wheat species growing in Europe that may hybridise with cultivated wheat, the tetraploid wild emmer wheat (*Triticum turgidum* var. *dicoccoides*) found in Greece, although there certainly is evidence that such gene flow may occur elsewhere. For instance, genetic variation in wild emmer and tetraploid wheat (domesticated emmer) in Israel indicates ancient gene flow (Huang *et al.*, 1999), and the authors considered it likely that gene flow still occurs between them.

Another close relative of wheat, jointed goatgrass (*Aegilops cylindrica*), can be found as a weed in cultivated fields of southern and eastern Europe. This species shares the D-genome with cultivated bread wheat. In the United States where *A. cylindrica* is a serious weed, spontaneous herbicide-resistant hybrids have been reported between this species, and non-GM herbicide-tolerant wheat (Guadagnuolo, 2001a,b). The hybrids have low fertility (Wang *et al.*, 2001), but backcrosses to *A. cylindrica* occur spontaneously (Mallory-Smith & Snyder, 1999; Morrison *et al.*, 2002). These observations therefore suggest that in Europe (trans)genes may be transferred to the weed in the event of commercial release of GM wheat. Gene flow may also be possible from wheat into more distantly related species in Europe. For instance, Guadagnuolo *et al.* (2001a) surveyed English and Austrian populations of wild *Hordeum marinum* and found some *Triticum*-specific markers, perhaps indicating intergeneric gene flow and introgression from wheat.

5.4.2 Barley, *Hordeum vulgare* (10 600 000 ha)

Barley is strongly autogamous, with a very limited interpopulational gene flow (Ennos, 1994). Transgene flow between neighbouring barley plots has, however, been documented (Ritala *et al.*, 2002), making gene flow between GM varieties with different engineered modifications a likely eventuality. Outside the cultivated forms, fertile hybrids are easily produced between cultivated barley *H. vulgare* ssp. *vulgare* and the wild, weedy form of the same species, *H. vulgare* ssp. *spontaneum*. Thus, spontaneous gene exchange is likely in areas where both forms are found together (Bothmer *et al.*, 1995). In Europe, ssp. *spontaneum* is distributed in the eastern part of the Mediterranean and so this is where hybrids can be expected (Bothmer *et al.*,

1995). Barley is wind-pollinated and so the abundance of infraspecific hybrids will depend heavily upon the pollen dispersal characteristics. This can be measured by volumetric spore traps or else by direct observations of hybrid frequency. Ritala *et al.* (2002) adopted the latter approach to measure pollen movement from plots of transgenic barley. They used male sterile barley plants as trap plants that were positioned at various distances from the transgenic source. In 2 years of field trials, they observed that at 1 m distance 2–5% of the seeds were fertilised by the transgenic line. At a distance of 50 m from the transgenic source, cross-fertilisation was reduced to 0.1–0.2%. These frequencies are naturally assumed to be much lower when self-pollen is available, but the work has value in indicating the pattern of pollen decline with distance. There are strong crossing barriers between cultivated barley and the wild barley species (Bothmer *et al.*, 1995) and so spontaneous interspecific gene flow is currently viewed as highly improbable although not impossible given the large numbers of plants involved.

5.4.3 Maize, *Zea mays* (4 350 000 ha)

Maize is monoecious, wind-pollinated and outcrossing. Various studies have indicated that pollen flow occurs from elite varieties to landraces and also between varieties (Doebley, 1990; Sanou *et al.*, 1997). The significance of such genetic exchanges is dependent upon context, with the greatest concern being shown over the possibility of gene flow to organic farms and to landraces. For instance, Quist and Chapela (2001, 2002) demonstrated the latter when they reported the presence of transgenes in Mexican landraces of maize. This gene flow was probably a result of erroneous or unauthorised cultivation of Bt maize in the area. Interspecific gene flow is also a possibility in parts of the Americas, particularly to a progenitor of maize, teosinte (Doebley, 1990), although there are no wild relatives of maize in Europe. Collectively, therefore, the scope for gene flow from maize to these various recipients has heightened interest in the possibility of invoking some form of risk management. Garcia *et al.* (1998) argued that the monoecious nature of maize is useful in this respect, suggesting that genetically modified maize should be detasselled to prevent dissemination of novel genes into sexually compatible landraces. The commercial practicality of such a measure is questionable. The imposition of isolation distances is a simpler alternative but is heavily dependent on airborne pollen distribution profiles. There have been several studies aiming to characterise pollen dispersal profiles from maize, but these have largely been performed using small-scale donor plots (e.g. Luna *et al.*, 2001; Jarosz *et al.*, 2003). In a review of these studies, Emberlin *et al.* (1999) reported that airborne pollen densities at the plot or field margin typically declines to 2% at 60 m but remains at 0.5–0.75% at 500 m. The actual quantity of pollen at such distances depends on the size of the emitting crop, the presence of intervening physical barriers and wind strength (Ingram, 2000), but Emberlin *et al.* (1999) suggested that at 500 m, 125 000 pollen grains may still be airborne from an individual plant. They calculated that under average conditions, with synchronous flowering and with donor and recipient plots of equal

size, this roughly translates to approximately 1 kernel per 135–204 set on a recipient plant. The UK Advisory Committee for the Release into the Environment (ACRE) later evaluated the regulatory significance of this work. They concluded, ‘ACRE accepts that pollen may be carried on air currents for great distances, but *pollen dispersal* is not the primary issue. The main consideration is the frequency with which that dispersed pollen results in *successful hybridisation* (cross-pollination) at various distances from source. In giving its previous advice on the likelihood of cross-pollination of organic sweetcorn by GM fodder maize the Committee referred to internationally recognised data on cross-pollination frequency (UK Seeds Regulations, EC Seeds Directive and OECD Maize Seed Scheme) used to ensure high purity in maize seed production’ (ACRE, 1999).

To ensure that maize seed stocks achieve 99.9% purity, an isolation distance of 200 m is required from any source of contaminating maize pollen. This figure of 200 m is based on practical field experience of seed inspection authorities over many years and under a variety of environmental conditions. There may be cases where unusual prevailing weather conditions have led to more cross-pollination at 200 m than expected but practical experience shows these to be rare. This case illustrates with some clarity the need of the regulators for distilled information that aids the decision-making process (see Chapter 7). This is not to say that further information on gene dispersal is not necessary or desired. For instance, Chilcutt and Tabashnik (2004) evaluated the extent of gene flow from GM Bt maize and found a moderate incidence of Bt kernels (up to 45%) in non-Bt refuge maize plants required under the US Environmental Protection Agency (EPA) regulation to prevent the evolution of resistance among target pests. They concluded from their findings that guidelines should be revised to reduce gene flow between the GM Bt crops and the refuge plants. Similarly, the goal of producing spatially explicit models of gene flow between maize fields would be useful in predicting the extent of pollen-mediated admixtures of harvested seed under different scenarios of GM acreage. Several authors have made preliminary models that go some way towards achieving this aim (e.g. Klein *et al.*, 2003; Richter & Seppelt, 2004).

5.4.4 Pumpkins for fodder, *Cucurbita pepo* (5 100 000 ha)

Pumpkins are insect-pollinated. Studies from Mexico and the United States have suggested that gene flow has taken place between the crop and weedy types of the species (Wilson, 1990; Wilson *et al.*, 1994; Decker-Walters *et al.*, 2002). True weedy or wild types are not recognised in Europe, but intervarietal gene flow is a possibility. Other species of *Cucurbita* are cultivated especially in southern Europe.

5.4.5 Olives, *Olea europaea* (4 300 000 ha)

Olive is a wind-pollinated obligate outcrossing species and the seeds are dispersed by birds (Ouzzani *et al.*, 1993). This means that there is a strong potential for long-distance gene flow between GM varieties, and from GM varieties to wild olives.

Wild olives grow in the Mediterranean countries and they are closely related to, and compatible with, the cultivated olives (Ouzzani *et al.*, 1993). Besnard *et al.* (2001) suggested that gene flow could be responsible for the genetic differentiation between Mediterranean olives and olives from Asia and Africa. However, to date there has been no formal study attempting to quantify the extent of gene flow between cultivated olives or between these and wild populations of the species.

5.4.6 Oilseed rape, *Brassica napus* (3 500 000 ha)

Oilseed rape (rapeseed) is partly outcrossing (Becker *et al.*, 1992) and pollinated by insects and wind (see Chapter 3). In Europe, there are several wild species that are closely related to *Brassica napus*. Among these relatives *Brassica rapa* (= *B. campestris*) is the most likely recipient of oilseed rape genes (Chèvre *et al.*, 2004, Jørgensen *et al.*, 2004). *B. rapa* is a common weed in oilseed rape fields in parts of Europe and the Americas and also occurs along field margins and as stable, natural populations on riverbanks, for example in the United Kingdom (Scott & Wilkinson, 1998; Wilkinson *et al.*, 2003). Interspecific hybridisation is a rather common event when *B. rapa* is a weed in oilseed rape fields (Landbo *et al.*, 1996), but it seems that further interspecific introgression mostly occurs in places where weed control is inefficient, as the F₁ hybrids especially are apparently vulnerable to weed control practices (Hansen *et al.*, 2001, 2003). These authors revealed a high frequency of spontaneous gene flow to an advanced generation of crosses between oilseed rape and *B. rapa* in a large weedy population of the two species in an organic field. Genetic analysis of the plants suggested that the oilseed rape DNA had been recombined into the genome of *B. rapa*. It was also demonstrated that transgenes engineered into the plastid DNA of oilseed rape can disperse to the weedy *B. rapa* through crosses in which oilseed rape acted as the female parent.

The recruitment of transgenes in *B. rapa* was similarly confirmed in field experiments with transgenic herbicide-resistant oilseed rape. Mikkelsen *et al.* (1996) performed a detailed survey of transgene movement from GM HT oilseed rape into weedy *B. rapa*, and reported spontaneous hybrids and subsequent introgression such that in two plant generations, transgenic offspring were obtained with a chromosome number and fertility corresponding to the weedy species. Furthermore, later field experiments showed that a variety of fitness parameters exhibited by the non-modified hybrids and backcross plants could be as high as and even higher than those of the weedy parent (Hauser *et al.*, 1998a,b, 2003). Collectively, these data suggest that hybrids will be frequent in weedy populations and will almost certainly lead to introgression. The physical separation of oilseed rape from the natural riverside populations of *B. rapa* means that hybrid abundance and transgene recruitment rates will be very much lower here. Scott and Wilkinson (1998) provided the first quantitative measure of spontaneous hybrid seed formation between oilseed rape and riverside populations of *B. rapa* in southern England. They found that seeds taken from sympatric *B. rapa* river populations 1–8 m from commercial fields of oilseed rape included 0.4–1.5% hybrids. It does not follow, however, that the abundance of

hybrid seeds necessarily equates to the number of hybrid plants, since hybrids may show either depressed or enhanced fitness relative to the resident *B. rapa* plants, or may exhibit reduced seed dormancy (Landbo & Jørgensen, 1997; Linder, 1998). In addition, data such as these provides little indication of the numbers of hybrids expected within any given geographic region. Wilkinson *et al.* (2000) provided the first study that attempted to address this problem by using satellite imagery and digital river systems information to identify riverside fields of oilseed rape across 1500 km² of southern England. These fields, representing potential sites of sympatry, were then each visited; only two were found to be adjacent to riverside populations of *B. rapa*. Only one hybrid plant was found in one site out of the 505 plants present in the two populations. This approach was then adapted and extended in a more recent study (Wilkinson *et al.*, 2003) to produce a national estimate for hybrid frequency between these species arising from local hybridisation events. Nationally, 26 000 ($\pm 22\ 000$) locally formed hybrids were predicted annually in riverside populations adjacent to oilseed rape, with extensive regional variation. Long-range hybrids were modelled at around 6000 p.a. on the basis of pollen dispersal curves. This type of study provides regulators with starting points from which to evaluate risk management measures that attempt to remove the exposure element (i.e. measures to prevent hybrid formation) and for more ambitious models to predict the rates and pattern of gene introgression. Equally, they can be used to designate areas of highest gene flow for post-release monitoring efforts.

Progress on other relatives has been more modest. For instance, spontaneous gene flow from transgenic oilseed rape to *Raphanus raphanistrum* has been shown in field experiments (Chèvre *et al.*, 1997, 1998, 2003), but the transgene was not recombined into the genome of the weedy species. Apparently, gene flow from oilseed rape to other weedy relatives such as *Sinapis arvensis* or *Hirschfeldia incana* (Lefol *et al.*, 1996a,b; Chèvre, 2003) will be a very rare event.

There is also considerable interest in intraspecific gene flow among fields of oilseed rape. Clearly, there is the capacity for long-range pollen delivery over several kilometres either by wind (Timmons *et al.*, 1996; Thompson *et al.*, 1999) or by insect vectors (see Chapter 3). The key issue, however, is the extent to which pollen-mediated gene movement between GM and non-GM fields will create admixtures in the harvested seed (potentially reducing the value of the latter). Moreover, experience from large-scale cultivation of herbicide-resistant genotypes in Canada (Downey, 1999; Hall *et al.*, 2000; Beckie *et al.*, 2003) demonstrated that resistance to different herbicides – encoded by different transgenes – had become stacked in volunteer oilseed rape plants as a consequence of gene flow between neighbouring fields with different types of resistance. Difficulty now lies in anticipating the extent and scale of such gene flow on a landscape scale (see Chapter 3).

5.4.7 Vine, *Vitis vinifera* (3 500 000 ha)

Grapes are predominantly self-pollinating, with limited outcrossing mainly performed by insects. The cultivated vine is derived in part from selection from the

wild subspecies *ssp. sylvestri*. The exact distribution of the wild subspecies is unknown although it is found in eastern and central parts of Europe. Some of the wild vines are probably recent hybrids between the wild and cultivated subspecies (*ssp. sativa*) (Sefc *et al.*, 2003). Other species of *Vitis* have been introduced to Europe from North America and their interspecific hybrids with *V. vinifera* are also cultivated. Thus, while gene flow is certainly a possibility, there is virtually no basis on which to predict the extent or pattern of genetic exchange expected in the event that a GM grape variety is released commercially.

5.4.8 *Sunflower seeds, Helianthus annuus (2 200 000 ha)*

Sunflower is habitually outcrossing and is mainly pollinated by honeybees. In the United States, cultivated sunflower fields are often infested by or adjacent to its conspecific wild relative, common sunflower (*Helianthus annuus*). Indeed, Burke *et al.* (2002) found that approximately two thirds of cultivated sunflower fields occurred in close proximity to the wild relative and found evidence of hybridisation in 10–33% of these wild populations. There is also scope for longer range hybridisation between these plants, with hybrids reported up to distances of 1000 m (Arias & Rieseberg, 1994). Thus, it seems that extensive isolation distances would need to be imposed if gene flow is to be avoided between these taxa. The ecological significance of these hybrids to some extent rests on the fertility of the hybrids and the stability of crop DNA in the genome of the wild relative. Whitton *et al.* (1997) tested this by following the transmission and stability of two cultivar-specific markers in 2700 progeny and in four subsequent generations. They found that hybrid frequency was high (42% at the donor plot margin) and did not fall over the ensuing generations. It seems plausible therefore that both hybridisation and introgression will occur from GM sunflower into wild *H. annuus* populations in the United States. Furthermore, from their study of wild sunflowers with Bt transgenes providing insect tolerance, Snow *et al.* (2003) concluded that Bt genes are likely not only to spread to wild and weedy populations but also to increase seed production in the wild populations when these herbivores are common. These data are sufficient for the regulators to adopt a working assumption of widespread gene flow in the United States and so to focus attention on the consequences arising from the transfer of specific transgenes. In contrast, there are no reports of wild relatives of sunflower in Europe. Here, effort should centre on quantifying the capacity of pollen-mediated gene flow between GM and non-GM fields of the crop and between the former and feral or volunteer populations of cultivated sunflower.

5.4.9 *Sugar beet, Beta vulgaris ssp. vulgaris (2 000 000 ha)*

Cultivated beet is wind-pollinated, though a few insects can carry beet pollen around (Free *et al.*, 1975). The cultivated types are primarily triploid and are usually harvested before flowering. However, a few plants in most fields do bolt and flower before harvest and these are apparently able to outcross or produce a few seeds by

selfing. For commercial seed production, male sterile female plants are sired by pollen-producing lines. The pollen donors are rather self-incompatible but seeds can be set as response to environmental stimuli or late in the season. Several interfertile wild and weedy forms coexist within the species: sea beet (*Beta vulgaris* ssp. *maritima*), which is an obligate outcrosser (Bruun *et al.*, 1995), weed beet and inland beet (Desplanque *et al.*, 1999). It is primarily in the seed production areas that hybridisation takes place. In several of the European beet cultivation areas hybridisation between cultivated and wild or weedy beets has been documented (Bartsch & Schmidt, 1997; Desplanque *et al.*, 1999; Vigouroux & Darmency, 1999; Andersen *et al.*, submitted; Viard *et al.*, 2004). The inland beets have a dominant gene for early flowering (Boudry *et al.*, 1994) and this character will therefore be inherited by hybrids between sugar beet and inland beet. If such hybrid seeds are exported with cultivated beet seeds, there will be the possibility for gene flow from these hybrids to wild relatives in areas where beets are grown for production.

Detailed recent studies have shown on a regional level that hybridisation is extensive and that it is both pollen- and seed-mediated (Arnaud *et al.*, 2003; Viard *et al.*, 2004). Emphasis now needs to turn to the efficiency of introgression and to identify regions of the genome where introgression is repressed or enhanced.

5.4.10 Oat, *Avena sativa* (1 900 000 ha)

Oat is mostly cultivated in northern Europe. The crop hybridises rather easily with *A. fatua*, a common weed in cereal crops, but also with the rare *A. sterilis*. Frequencies of spontaneous hybridisation with *A. fatua* were from 0 to 1% in Australian fields (Burdon *et al.*, 1992), apparently depending on the relative proportions of the parental species in the mixed stand. Frequencies of hybridisation under European conditions are unknown.

5.5 Methods for detection and quantification of rare hybrids

It is quite evident then that there is considerable variability in the extent to which GM crops will be able to form hybrids with non-GM varieties or wild and weedy relatives. It is also clear that there is a significant variation in the state of our current knowledge about gene flow rates expected for different crops in different regions. It appears that thus far progress has been rather idiosyncratic, and dependent partly on the local legislative priorities but also on the interests of the rather small numbers of scientists that are active in this area. Even for data relating to the initial hybrid formation, the level of information available ranges from simple crossing experiments that aim to demonstrate that hybrid formation is possible, through the reporting of spontaneous hybrid seeds and then plants, their quantification in a natural context and eventually to their spatial quantification on a landscape scale. Progress on the characterisation of introgression has been less impressive, with almost all studies relying on the presence of unmapped anonymous markers and no information available on how

integration site affects transmission rates. However, as the technology is applied to more crops, so the need for this kind of information will also increase. It is therefore important that a hierarchy of techniques develop to generate the required information as quickly and effectively as possible. The procedures needed for the detection of rare hybrids provide the greatest challenge. While others have described the array of methods that can be used to detect hybrids (e.g. Kjellsson *et al.*, 1997; Westman & Kresovich, 1997; Auer, 2003; Ellstrand, 2003), the aim here is to suggest a systematic approach for progressively generating information of relevance for regulation (see Chapters 7, 9 and 10).

In essence, the first requirement for any risk assessment relating to gene flow is the specification of the possible recipients of transgenes from a particular GM crop in a defined geographic region. In the absence of wild or weedy relatives, this task is relatively simple: concerns of gene flow will relate only to other fields of the crop and feral/volunteer populations of the crop. In many cases, however, the list of possible recipients is either absent or incomplete, most frequently because of uncertainty over the cross-compatibility of the crop with the putative recipient taxon in question. Under these circumstances, a series of artificial crossing experiments would be required, ideally using a representative diversity of the recipient and crop as parents. Emphasis then turns to the task of attempting to quantify hybridisation rates. Initially, this is most readily addressed using a simple and direct series of field experiments in which the relative proportions of donors and recipients are manipulated to provide reasonable simulations of conditions in the natural environment. Such data has value in providing a rough guide to the numbers of hybrid seeds formed but may not reflect the abundance of hybrid plants since hybrids may differ from either parent in their fitness or seed dormancy characteristics. This is particularly true for relatives occupying natural or semi-natural habitats. While it may then be appropriate to estimate hybrid plant numbers in settings that can be adequately simulated in field experiments (e.g. that of agricultural hybrid weeds), more generally, it will become desirable to seek spontaneous hybrids in the field. The aim here is to provide qualitative information demonstrating survival of the hybrids in the wild but also to assess what the scale of the hybrid formation is likely to be under natural (not simulated) conditions. It is here that the task can become onerous when hybrids are rare, and so a system must be introduced to maximise the chances of success. First, it is important to identify several geographically dispersed sites in which donor and recipient are growing in maximal proximity to each other. For weedy relatives, this may be a series of heavily infested fields of the crop, whereas for recipients in natural habitats it will be sites where the agricultural fields are close to the recipient habitat. Having identified the sites, hybrid frequency is probably most easily assessed by collecting seeds from the recipient (and/or the crop) and then screening the resultant offspring under controlled conditions. The use of controlled growth conditions minimises phenotypic variation attributable to environmental perturbations and so improves the reliability of screening on the basis of appearance. Identification of hybrid plants in the field can be more problematic and will generally require collection of large quantities of leaf material for subsequent

molecular analysis. Whichever source material is used, the search for hybrids usually requires a two-stage process: a preliminary screen in which putative hybrid plants are identified and a confirmation assay in which the putative hybrids are confirmed as such on the basis of a more robust assay. The choice of methods for each of these stages will depend on the crop, the laboratory and the frequency of the hybrids. We outline some possible options below.

5.6 Preliminary screens for hybrids

5.6.1 Morphology

Morphological character analysis, i.e. analysis of plant phenotypes, is an obvious possibility for detection of rare hybrids, provided that morphological differences distinguish the parental species and the hybrids share some of the characteristics of both species. In some cases, however, hybrids may appear indistinguishable from one parent or exhibit widely variable phenotypes that range between those of its parents. For instance, in controlled crosses between *Brassica tournefortii* and *B. rapa*, Choudhary and Joshi (2001) found that the F₁ hybrids were intermediate to their parents for most of the morphological traits but a few characters were inherited selectively from the maternal or the paternal parent. This skewed distribution of phenotypic traits has also been observed following hybridisation in other genera, for example in interspecific hybrids of *Cucumis* (Chen *et al.*, 2004).

When using phenotype as a preliminary screen, ideally plants should be grown as a cohort and examined regularly. A small number of qualitative features are generally preferable to the more time-consuming process of collecting several complex measurements. In this way large numbers of plants can be examined in a relatively short time frame. For example, Scott and Wilkinson (1998) used leaf pruinosity, colour and hairiness to screen through 13 000 seedlings collected from *B. rapa* plants growing next to fields of oilseed rape. They found just 46 hybrids.

In some cases, however, it may not be possible to grow fresh material, or interest lies in identifying hybrid plants in the field. The morphological analysis can then be used *in situ* or on dried herbarium material and only requires standard laboratory equipment and use of appropriate statistical programs. Good descriptions of methods for morphological analysis are provided, for example, by Kjellson *et al.* (1997) or Knox *et al.* (1995). Data collected from herbarium specimens sometimes preclude molecular analysis and in these instances should be processed by some kind of multivariate statistics (e.g. PCO or PCA) for detection of the hybrids and introgressed plants. Hauser and Bjørn (2001) described spontaneous hybridisation between cultivated and wild carrot using morphological characters in this way.

Ideally, morphological identification of hybrids should not be used in isolation but should be combined with other methods for hybrid identification as there are often few morphological characters differing between taxa, and the genetic background of

these characters is usually complicated, unknown and modulated by the environment (Rieseberg & Wendel, 1993).

5.6.2 Sterility

Hybrids may show a decreased fertility compared to their parental genotypes. This can sometimes be exploited for the purposes of provisional hybrid identification. Seed production or seed viability can be a good indicator of hybridity as seed production per flower is often reduced. Reduced pollen fertility is of less value, largely because of the need for microscopic examination, although it can be a useful additional indicator for plants showing other signs of hybrid status (reduced seed set, intermediate morphology). Pollen viability can be evaluated by different viability stains or more simply by observations on pollen size and shape to identify the frequency of misshapen grains. Methods for estimating pollen fertility are described in most textbooks on staining procedures in biology. Jørgensen and Andersen (1994), Hauser *et al.* (1998a,b, 2003) and Pertl *et al.* (2002) report on male and female fertility in hybrids between oilseed rape and *B. rapa*.

5.6.3 Herbicide bioassays and visible marker genes

Transgenes – at least when they represent novel traits – are the perfect tools to perform preliminary screens for hybrids or introgressed individuals. The most widely grown GM herbicide-tolerant plants are tolerant to the herbicides glyphosate or glufosinate but bromoxynil- and acetolactate synthase (ALS)-inhibitor-tolerant GM plants have also been developed. Herbicide bioassays can be carried out by the germination of seeds on herbicide-containing medium or filter paper as, for example, performed by Pfeilstetter *et al.* (2000) for Basta (glyphosinate) tolerant oilseed rape. Spray tests in the laboratory and in the field are also quick methods to detect the herbicide genes. Mikkelsen *et al.* (1996) detected spontaneous transfer of a herbicide tolerance gene from oilseed rape to *B. rapa* by Basta spraying, and Hall *et al.* (2000) showed transfer of multiple herbicide resistance genes to oilseed rape volunteers in spray tests. In oilseed rape, Rieger *et al.* (2002) showed the intraspecific dispersal at the landscape level of a gene-encoding tolerance to an ALS inhibitor spraying by the offspring; the herbicide-tolerant oilseed rape was produced by traditional breeding and not through genetic modification. Pfeilstetter *et al.* (2000) compared different screening tests using the Basta spray test, the drop test, ELISA (enzyme-linked immunosorbent assay-) screening and PCR (polymerase chain reaction) amplification, and found good correspondence between the results from the different types of tests.

Visible marker genes engineered into GM plants can also be targets of identification. The β -glucuronidase (GUS; Gilissen *et al.*, 1998) that is detected by a histochemical procedure and the green fluorescence protein (GFP; Stewart, 2001; Hudson *et al.*, 2001) are examples of such markers. In GM plants where an antibiotic resistance such as the NPTII (neomycin phosphotransferase II) gene is inserted, selection of the tolerant plants is possible by adding the antibiotic agent to the growth

medium. However, present EU regulation is aimed at phasing out the antibiotic resistance markers in the production of GM plants.

5.6.4 Protein assays

When dealing with gene flow from transgenic plants, the transgene product can be identified in the recipient. The methods can be based on antibodies that are specific against the new proteins that are produced in the plants. Presently, commercial methods are available for Bt toxin and for herbicide tolerance. These lateral flow strip tests are cheap and can be used on site for detection but not quantification. However, as a result of operator performance, false negatives seem to be frequent (Fagan, 2004). The ELISA is more sensitive and can in principle (but with uncertainty) be used for quantification. Stave (1999) described the quantitative ELISA detection of Roundup Ready[®] soybean. Nevertheless, these tests are useful tools for preliminary hybrid screens.

5.6.5 Chromosome analysis and flow cytometry

Analyses of chromosome number, chromosome pairing and/or chromosome morphology are methods that have been widely used in scientific studies for the detection of hybrids. However, approaches differ in their suitability for large-scale hybrid screens. Many polyploidy crops have predominantly diploid wild relatives (e.g. potato, *Solanum tuberosum*; wheat, *Triticum aestivum*; oilseed rape, *Brassica napus*) so that interspecific hybrids typically have intermediate ploidy level. Flow cytometry is a fast method for quantification of DNA contents, allowing identification of hybrids between two parentals that differ in their cell contents of nuclear DNA. Often there is a correlation between the chromosome number and the DNA contents of the cell. The analysis demands access to a flow cytometer that analyses the degree of fluorescence associated with the nucleus in a labelled population of cell nuclei. This is achieved by passage of the nuclei one by one through a flow cell (at maximum rates over 1000 cells/s) where the stream of nuclei intersects a laser beam where they absorb light, which is subsequently re-emitted in the form of fluorescence. The emitted fluorescence comprises a rapid series of pulses that are converted into DNA amounts. The nuclei of plants with known DNA contents are used as control plants. In separating diploids from triploids, tetraploids, etc., the method has great potential; however, the sensitivity of the method is in many cases not sufficient to verify the gain or loss of one or a few chromosomes. Flow cytometry has been applied in detection of spontaneous hybrids, for example, spontaneous hybrids between oilseed rape (*Brassica napus*) and *Raphanus raphanistrum* (Darmency *et al.*, 1998) or *B. rapa* (Wilkinson *et al.*, 2000) and hybrids between cultivated beet and *Beta vulgaris* ssp. *maritima* (Andersen *et al.*, in press).

A more accurate but time-consuming approach is to count chromosome numbers from root squash preparations. This strategy has special utility when the aim is to identify introgressed individuals as well as F₁ hybrids themselves. In hybrids where

contributing parental genomes are not homologous, the pairing of chromosomes during the meiotic cell division can be irregular. This may result in the formation of univalent or multivalent chromosome structures. Methods for the preparation of chromosomes for counting or meiotic analysis are described in most textbooks on cytology (e.g. Fukui & Nakayama, 1996). Several authors have used these approaches to identify spontaneous hybrids and introgressed offspring between the tetraploid oilseed rape (*B. napus*) and the diploid relatives *B. rapa* and *Raphanus raphanistrum* (Jørgensen & Andersen, 1994; Chèvre *et al.*, 1997, 1998; Hansen *et al.*, 2003). Chromosome numbers of other spontaneous hybrids are reported in several floras, for example the flora by Stace (1975) on hybrids of the British Isles.

Genomic in situ hybridisation (GISH) offers a powerful means of characterising the nature and origin of a hybrid in instances where parentage or genome structure are uncertain, or of introgressed individuals, although the protocol is time-consuming and ill suited for screening purposes. There are many examples in which GISH has been used to characterise hybrids between crops and their wild relatives. For example, Ananthawat-Jonsson and Thorsson (2003) used GISH to characterise spontaneous interspecific hybrids in *Betula*, and the hybrid origin of the *Elymus* and *Elytrigia* species were verified by GISH analysis (Orgaard & Ananthawat-Jonsson, 2001).

5.6.6 *Microarrays*

Currently, microarrays (biochips) for GMO identification are marketed only by a few companies. DNA microarrays/biochips consist of multiple captured probes grafted onto a surface. The probes are chosen to be complementary to the target sequence that is going to be detected. Each captured probe will bind to its corresponding target sequence. At present, the chips allow for identification of engineered genes on an individual plant basis, but in the long run the purpose of the chips is to detect many genes present in a sample in one assay rather than performing individual gene assays. In this way it will be possible to test for all GM crops approved and so could be used for very large-scale screening of populations for rare hybrids. More information on biochips is available from the EU project aimed at developing the technique in food (www.gmo-chips.org).

5.7 **Confirmation of hybrid status**

5.7.1 *Isozymes*

Isoenzyme analysis has been widely used to identify hybrids as it is relatively cheap and quick. Moreover, genetic information is often present on the loci studied, which together with the codominant expression facilitates the interpretation of the phenotypes. The method builds because enzymes have a different electric charge and catalytic ability and therefore they can be separated and visualised in an electric field over gels made of, for example, starch or polyacrylamide. The visualisation is carried

out by immersion of the gel in the substrate of the enzyme with a subsequent staining of the product of the enzymatic reaction. Enzyme phenotypes are then revealed as bands on the gel. Not only isoenzymes (different alleles from the same enzyme locus) but also proteins in general may be visualised by applying protein specific stains. Darmency and Gasquez (1997) identified the possible parents of *Poa annua* from isoenzyme profiling of spontaneous hybrids, and detection of spontaneous hybrids between oilseed rape and *B. rapa* was carried out by isozyme analysis by Jørgensen and Andersen (1994). The endosperm storage proteins, high molecular weight (HMW) glutenins, were used as genetic markers for identifying hybrids between cultivated wheat and jointed goatgrass (Morrison *et al.*, 2002). For a more detailed description of the methods for protein profiling, see the review by Kjellsson *et al.* (1997) and Doebley (1989).

5.7.2 DNA-based methods

The most widely used methods for the confirmation of hybrid status are DNA-based marker techniques. The PCR-based markers such as SSR (microsatellites), AFLP, SSAP and RAPD are especially popular because the methods are quite easy to perform and because they quickly provide taxon specific markers for identification of intertaxon hybrids. Furthermore, these markers are considered selectively neutral, although they may be linked to genes that are subject to selection. The DNA under study can be derived from the nucleus or from the cytoplasmic organelles. Under some circumstances, the pooling of DNA from several individuals allows some of these approaches to be used for screening purposes. The pooling of tissues from different individuals prior to extraction can offer an enticing alternative but runs the risk that not all individuals are equally represented and so pools need to be modest-sized to avoid the risk of false negatives. A range of possible molecular procedures that have been used for the identification of rare hybrids is listed below.

5.7.2.1 PCR-based techniques

The PCR method is an amplification of previously identified DNA sequences by simultaneous primer extension of the complementary DNA strands. Two specifically designed primers are needed, each bordering the target sequence. The primers are short oligonucleotides (usually 18–23 base pairs). The primers are added to the plant DNA and they act as templates for amplification of the target DNA. Amplification is carried out by a thermostable polymerase in the presence of the four DNA nucleotides. After the reaction, the amplified target sequences can be separated on gels and visualised by various DNA stains. Information about the transfer of a target sequence from a donor to a recipient can be backed up by the inverse PCR technique that allows amplification and thus subsequent sequencing of the DNA that is flanking the core region of the known sequence. The PCR technique has been carefully described in several books and articles, for example by McPherson *et al.* (1991). A range of molecular strategies based on PCR that have been used for hybrid confirmation is described below.

5.7.2.2 *Random amplified polymorphic DNA*

The random amplified polymorphic DNA (RAPD) method was the first PCR-based approach to be widely used for hybrid verification. The method uses arbitrarily selected primer sequences to generate multiple products by PCR. The RAPD technique has been described in more detail, for example, by Rafalski and Tingey (1993) and Kjellsson *et al.* (1997). The complex array of amplicons are generally separated on the basis of size by agarose gel electrophoresis and visualised using a DNA-specific stain such as ethidium bromide. RAPD analysis is fast, cheap and usually produces sufficient polymorphisms to allow for the identification of most hybrid types. The amplicons in the multiple band profiles are anonymous (homology is inferred only on the basis of co-migration) and are usually inherited in a dominant fashion, which can limit its usefulness for hybrid verification. Furthermore, while careful laboratory practice allows for reliable results within a laboratory, reproducibility between sites is generally not possible. For these reasons, the technique has recently fallen out of favour, with more powerful alternatives listed below being preferred. Nevertheless, the technique has proved useful for hybrid confirmation in the past. For instance, Jørgensen and Andersen (1994) revealed spontaneous hybridisation between oilseed rape and *B. rapa* using RAPD markers, and Isoda *et al.* (2000) detected spontaneous interspecific hybridisation in *Abies* by way of RAPD markers.

5.7.2.3 *Amplified fragment length polymorphism*

Amplified fragment length polymorphism (AFLP) is a more powerful PCR-based DNA fingerprinting technique than RAPD. It is based on selective amplification of a subset of restriction fragments from a digest of DNA (or sometimes cDNA), with subsequent visualisation of the PCR products on a gel (for full details, see Vos *et al.*, 1995; Kjellsson *et al.*, 1997; and Mueller & Wolfenbarger 1999). Usually the method generates very complex amplicon profiles comprising 50–100 products. As with RAPD analysis, the markers in these complex profiles are usually inherited in a dominant fashion. However, their complexity and reliability offers huge advantages for confirming the identity of hybrids and introgressants, potentially allowing designation of cultivar of origin and semi-quantification of the extent of introgression. There are several examples where AFLP has been used to confirm hybrid status. For instance, Hansen *et al.* (2001, 2003) used AFLP to confirm interspecific hybrids and introgressed progeny arising from gene flow between oilseed rape *B. napus* and *B. rapa*. Similarly, Quagliaro *et al.* (2001) used the AFLP technique to distinguish hybrids in sunflower.

5.7.2.4 *Simple sequence repeats (microsatellites)*

Simple sequence repeats (SSRs) or microsatellites are stretches of DNA that consist of tandem arrays of 2–8 base motifs. SSRs are abundant in all investigated eukaryotic genomes, and lead to a high level of intraspecific polymorphism for the number of repeat motifs within an array. Length variability within an SSR can be visualised by PCR analysis using primers that are specific to the flanking sequences of the SSR-locus, followed by high-resolution electrophoresis. The value of the SSR derives from its multiallelic nature, the co-dominant inheritance and the ease and reliability

of detection. The co-dominant nature of allelic inheritance is particularly useful for confirming inheritance of alleles from both parents in hybrids. However, the main disadvantages are that sequence information is required to design the flanking primers, and that the primer sets usually have to be developed separately for each species or species complex, which involves cloning and sequencing. One possible method is described by Kjellsson *et al.* (1997). Thankfully, however, SSRs are already available for most important crops (e.g. Saal & Wricke, 1999; Sharopova *et al.*, 2002; Holton *et al.*, 2002; Li *et al.*, 2003) and these can often be used on the crop's wild relatives. It is usually desirable to use several SSR loci to confirm hybrid identity, and the cost and time of analysis can be reduced if several SSR analyses are performed together. This is known as multiplexing and has been used widely, for instance in the study of oilseed rape (Tommasini *et al.*, 2003) and bean (Masi *et al.*, 2003). There are studies where SSR markers have been used for identification of interspecific hybrids, for example, in rice (Song *et al.*, 2003) and between cultivated beet and wild beet, *Beta maritima* (Andersen *et al.*, in press).

The ISSR (inter simple sequence repeats) method also targets SSRs but exploits their abundance rather than their variation in length. Like AFLP and RAPD, however, it produces complex profiles that represent many loci and exhibit dominant inheritance patterns. Nevertheless, this is a more simple technique than AFLP and offers similar levels of resolution, such that it is possible to determine intercultivar hybrids of oilseed rape (Charters *et al.*, 1996; Reddy *et al.*, 2002) or to identify the cultivar that gave rise to an interspecific hybrid between oilseed rape and *B. rapa* (e.g. Wilkinson *et al.*, 2000). Similarly, Ruas *et al.* (2003) used ISSR markers to unravel the parentage of coffee interspecific hybrids.

5.7.2.5 Quantitative PCR (real-time PCR)

Quantitative PCR (real-time PCR) is a very sensitive quantification of a specific sequence. This PCR method is based on the quantification of fluorescent reporter molecules that increase in proportion to the amount of PCR product in the reaction. When analysing the percentage of GM contents in seed lots and food, quantitative PCR has become the preferred technique. Wiseman (2002) and Levin (2004) described the state of the art and limitations of quantitative PCR. For GM maize, Hernandez *et al.* (2003) described the quantification of the MON810 construct providing tolerance to lepidoptera damage. Mellon and Rissler (2004) reported on quantitative detection of transgenic material in commercial seed lots of maize, soybean and canola. However, in principle, the approach could also be used for large-scale screening for rare interspecific hybrids using pooled DNA samples.

5.8 Introgression

Unequivocal verification of introgressive hybridisation can be difficult. Traits appearing as a result of introgression are hard to separate from primitive traits inherited from a common ancestor or from traits resulting from convergent evolution. Dealing with the flow of transgenes that encode totally new traits, this character ambiguity is

prevented and as such transgenes are the perfect markers of introgression. However, when transgenic markers are lacking, the most powerful way to detect introgression is by tracing linked markers, i.e. markers located close to each other on a chromosome. If a putative introgressed plant reveals multiple linked markers, linked in the same way as in the mapped genome of the potential donor plants, mutual ancestors or convergent evolution can be ruled out. Therefore, an excellent tool for verification of introgression is comparative linkage mapping, which requires the construction of genetic maps for the donor and recipient species. Such maps are most easily generated by the use of multilocus molecular systems such as AFLP, SSR, ISSR, etc. that provide many markers in a short time. Spontaneous introgression has been verified by comparative mapping, for example in sunflower (Rieseberg *et al.*, 1995, 1996).

5.8.1 *Data analysis for introgression*

Several computer programs for analysis of genetic data are available on the Internet. In the following section, a brief review is given of a few programs that are especially suitable for the identification of hybrids or introgressed plants. For more programs for population assignment and hybrid analysis, this homepage can be consulted: http://www.bio.ulaval.ca/louisbernatchez/links.htm#soft_pop_assign. Multivariate statistics such as PCO and PCA are found in several of the freewares. Consult, for example, the software PCAGen by Jerome Goudet, which works with co-dominant data and calculates PCA and Fst-value between populations (see <http://www.unil.ch/izea/software/pcagen.html>).

5.8.2 *AFLPOP*

AFLPOP (<http://www.bio.ulaval.ca/contenu-fra/professeurs/prof-l-bernatchez.html>; Duchesne & Bernatchez, 2002) is designed primarily to solve allocation of populations that are analysed by AFLP markers. Given an AFLP genotype and a set of population samples, AFLPOP assigns each of these populations the specific genotype it is most likely to belong to.

5.8.3 *New Hybrids*

New Hybrids (<http://ib.berkeley.edu/labs/slatkin/eriq/software/software.htm#NewHybs>) estimates the probability that genetically sampled individuals fall into each of a set of user-defined hybrid categories. The method is described in Anderson and Thompson (2002).

5.8.4 *Structure*

Structure (<http://pritch.bsd.uchicago.edu/software.html>) is a software package for using multilocus genotype data to investigate population structure. It can be used, for

example, to infer the presence of distinct populations, assigning individuals to populations, studying hybrid zones and identifying migrants and admixed individuals. It can be applied to most of the commonly used genetic markers, e.g. microsatellites. This method has been described by Pritchard *et al.* (2000).

5.9 Conclusions

Quantification of hybrid frequency and accurate characterisation of introgression are key components of the exposure for all hazards relating to gene flow. It is vital that information relating to these elements is collected in a systematic and progressive manner so that regulators are clear on the state of knowledge relating to a particular crop–transgene–location combination. Early experiments should provide evidence of the capacity for hybridisation and then progress to provide semi-quantitative predictions of the rate of hybridisation. Finally, hybrid abundance should be predicted on a landscape scale and regions where hybrids are most and least abundant should be identified. This allows for the appropriate positioning of preliminary field trials and specifies where post-release monitoring will be most effective. It also provides insight into the feasibility of risk management on the basis of exposure. It is during the later phases that there is a need for large-scale screening for spontaneous hybrid seeds and plants. For this, we advocate that recipient populations or seeds from them should be systematically screened for hybrids, particularly rare hybrids, using a two-stage approach. The initial screen may use morphological features, sterility, protein and biochemical assays for the transgene product, flow cytometry or even microarrays or quantitative PCR using pooled DNA samples. The second stage aims to verify the identity of putative hybrids selected in the preliminary screen. Various molecular approaches can be used for this purpose, but SSR–PCR and AFLP probably represent the currently favoured approaches. In the future, one may expect greater interest in techniques that exploit single-nucleotide polymorphisms (SNPs) such as pyrosequencing (e.g. Polakova *et al.*, 2003) and mass spectroscopy (Werner *et al.*, 2002). Finally, the most demanding task is to characterise the nature of introgression. This stage is still in its infancy as far as GM risk assessment is concerned but should exploit both molecular and morphological features to measure the extent (and ultimately the genomic position) of introgressed DNA into the recipient.

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6 Assessing the ecological fitness of recipients

Arthur E. Weis

6.1 Introduction

Recent research has established conclusively that flow of genes frequently occurs from crops to their sexually compatible wild relatives when the two grow in proximity (e.g. Arriola & Ellstrand, 1996; Bartsch *et al.*, 1999; Ellstrand *et al.*, 1999; Jenczewski *et al.*, 1999; Papa & Gepts, 2003). Thus, the answer to the rather simplistic question of whether there is a real likelihood of transgene introgression from GM crops into recipient natural populations is almost invariably ‘yes’, albeit qualified by location and context. The next question in risk assessment is far more challenging but nevertheless of central importance to the risk assessment process: ‘what will be the fate of the particular wild populations that receive specific transgenes?’

This is an important question because many of the existing and proposed GM crops contain transgenes that confer traits with the potential to be adaptive in recipient wild populations (Stewart *et al.*, 2003; Wilkinson *et al.*, 2003b). For example, the acquisition of herbicide resistance by troublesome weeds could increase their fitness and so their density in environments under prevailing agricultural practices. Likewise, it is entirely plausible that dramatic increases in insect or pathogen resistance might increase population densities in wild populations limited by herbivore pressure. It is equally possible that the introduction of some traits may allow a recipient species to escape its current ecological boundaries and so expand into new habitats or niches. For instance, the introduction of transgenes that enhance tolerance of abiotic stresses such as drought or soil salinity could enable a recipient halophobe species to expand its range into new, saline plant communities. Thus, there is scope for gene flow leading not only to irreversible changes to the ecology of a recipient within its existing habitat but also to an increased capacity to invade new habitats.

Several fates are possible when a GM crop containing such fitness-enhancing transgenes is first planted in proximity to a sexually compatible recipient population (Wilkinson *et al.*, 2003a). The outcome for any particular case will depend on many factors, such as the reproductive and ecological fitness of hybrids (and later-generation backcrosses), the degree of overlap in the flowering periods of crop, wild and hybrid genotypes, the size and spatial structure of the receiving populations and the degree and pattern of temporal variation in the selective environment. Conceptually, the population consequences of gene flow will play out in two stages: first is the introgression of the transgene from the crop into the wild genetic background, followed by its subsequent selective increase or elimination from the wild population (Lavigne *et al.*, 2004). During the first stage, gametes carrying the transgenes can arrive in the wild population through the movement of GM crop pollen or through

the presence of feral escapes of the GM crop. The subsequent union of crop and wild gametes yield hybrid seeds and, subsequently, plants within the recipient population. Alternatively, hybrid seeds can form within the crop itself following delivery of wild plant pollen, and these seeds can disperse into the site of wild population, possibly aided by farm practice. The presence of hybrids, even those displaying very low levels of fertility, means that transgene introgression is theoretically possible, and given past experience with natural systems, almost inevitable (see Chapter 4). The greater the rate of gene flow and the greater the hybrid fitness, the more likely it is that introgression will occur within the expected commercial lifespan of a GM cultivar. During the process, the selection will act not only directly on the transgene but also indirectly through the fitness effects of the crop genes that flow with it by gametic disequilibrium. Here, I use the term 'gametic disequilibrium' (Rice, 2004) as distinct to 'linkage disequilibrium' to emphasize that hybridization and non-random mating causes associations among loci even if they are not physically linked. Crop genes involved in the domestication syndrome, or crop chromosomal rearrangements that disrupt meiosis, are a sort of 'genetic baggage' carried by gametes containing transgenes that depress hybrid fitness. Although this baggage can initially mask the positive fitness effects of an adaptive transgene, or amplify the negative effects of a maladaptive one, independent assortment will rapidly break down the effects of gametic disequilibrium not associated with tight linkage and thereby increasing chances of transgene introgression. In the slightly longer term, all but the crop genes most closely linked to the transgenes (i.e. those showing linkage disequilibrium) will be removed by recombination. Thus, the position of the transgene (the identity of flanking crop genes) and the genetic background of the GM crop will both be important in determining the speed of introgression. During this time, selection favouring transmission of the transgene will probably be counterbalanced by selection against unfavourable crop genes. It follows that the likelihood of successful introgression will be a function of the broad level of genetic baggage (mediated by gametic disequilibrium), local genetic load associated with crop genes flanking the transgene (linkage drag), the fitness advantage conferred by the transgene and the frequency of recombination.

In this way, the introgression of crop genes into wild relatives is at this first step governed largely by internal selection, that is, by selection against genetic incompatibilities accumulated over the period of reproductive isolation between the parental taxa.

The second stage of the process (selective increase or elimination of the introgressed transgene) is governed by the selection imposed by the external environment on wild plants containing the transgene. The effect of the transgene on fully introgressed individuals among the recipient wild population will be determined by the strength of any fitness advantage imparted by the transgene when expressed in the wild-type genetic background in the wild-type habitat. Fluctuations in the local environment can alter the strength or even direction of selection of a transgene, and some of this variation in fitness can be frequency-dependent or density-dependent. The consequential effects of enhanced or depressed fitness will depend on context. For instance, a transgene conferring a significant ecological advantage could increase

the recipient's ability to displace other plant species (enhanced interspecific competitiveness), to invade new habitats (expanded niche range), to precipitate a decline in the herbivores and plant parasites that depend on the recipient (bivoltinid interactions) or to indirectly influence the abundance of fauna such as predators that interact with herbivores (multitrophic effects). On the other hand, a gene that increases only intraspecific competitive ability could induce a genetic sweep through the recipient without changing its abundance and density relative to the larger community, although the genetic diversity and structure could be affected. Thus, predictions on the implications of transgene spread require some knowledge of the demography of the receiving populations and of the interactions with other species in the community in which it resides.

The challenge to risk assessment will be to quantify the relative potential for each path that a particular crop–transgene–wild species complex can take. A complete assessment would require the integration of these many types of data into a quantitative model capable of generating predictions that would be phrased, for example, as 'there is a probability P that allele frequency of transgene Q will reach frequency R in wild populations that are S meters from cultivated fields within T years of commercial release' and 'introgression of allele Q is likely to result in a $U\%$ change in wild population density'. Generating such predictions is a daunting task at best, and perhaps impossible. It implies a level of predictive precision that ecological geneticists have seldom achieved, even in controlled microcosm environments stocked with genetically defined populations. To approach this level of precision, predictive quantitative models have to be tailored to the biology of the particular crop–relative system and parameterized with extensive data sets collected over a range of geographic location and environmental conditions. Nevertheless, there may be ways to integrate experimental results on the ecological and reproductive performance of crop–wild hybrids to help inform monitoring programmes (Kareiva *et al.*, 1996). Specific models from preliminary data can suggest what further experiments will be needed.

This chapter considers the potential impacts of gene flow from crops on receiving wild relatives. We will broadly define 'wild relative' to include entities ranging from volunteer stands of the crop species to self-sustaining populations of a different species, even in some cases species of a different genus. The following sections lay out some key elements in the biology of crop–wild hybridization that need to be parameterized for predictive models, that offer a glimpse on how such models are constructed, and that suggest priorities and necessary targeting when – as is likely – a comprehensive predictive model proves unfeasible for the foreseeable future.

6.2 Hybridization, introgression and internal selection

6.2.1 *The biology of natural hybrids and hybrid zones*

To understand the role of enhanced fitness in the initial stages of crop-to-wild introgression, it is necessary to consider what is known of introgression in natural populations. Plant species within the same genus do not regularly interbreed, which is precisely why they persist as identifiable species (Levin, 2002). Nevertheless,

hybridization is frequent enough to play an important role in plant evolution. An estimated 70% of all angiosperm species owe their origins to interspecific hybridization (Masterson, 1994). Ellstrand *et al.* (1996) examined published plant flora for mention of hybrid forms and found that interspecific hybrids are sufficiently frequent to be reported in 6–16% of plant genera, depending on geographic region.

Some of these liaisons are no doubt sporadic. However, persistent contact between sexually compatible populations can lead to formation of hybrid zones – geographical areas of overlap and hybridization between different taxa (Arnold & Hodges, 1995; Campbell & Waser, 2001). The classical view holds that the width and stability of these zones is maintained by a balance between selection against hybrids (primarily because of their low fertility) and the migration of parental types (gene flow) into the zone (Barton & Hewitt, 1985). This zone of low mean fitness thus creates a barrier to gene exchange, which is reflected in a cline in the frequencies of neutral alleles across the zone. In the extreme, depressed hybrid fitness can lead to selection for pre-zygotic isolating mechanisms. Contrary to conventional wisdom, however, hybrid fitness is not always low. Arnold and Hodges (1995) reviewed 44 studies on species that hybridize naturally, and compared fitness components of hybrids to parental taxa. In over half the cases, hybrids were as fit or fitter than the parental taxa. Looking at individual cases, some hybrid classes showed lower fitness than others (e.g. F_1 fitness lower than F_2).

How does low hybrid fitness impede the introgression of a given allele from one parental taxon to the other? When an alien pollen grain enters a receiving population, it carries not only the particular gene we might find of interest (such as a transgene) but also a variety of other alien genes, i.e. it is in gametic and linkage disequilibrium with alleles from the genetic background of the donor. For introgression to occur, the focal gene must be retained and recombined into the receiving genetic background quickly in order to avoid elimination by the selection acting against the alien background. Much theoretical work has been devoted to this question in order to understand the clines in neutral allele frequencies seen across hybrid zones (e.g. Barton, 1986; Barton & Shpak, 2000). Presenting the full mathematical results is beyond the scope of this chapter, but the important findings indicate that population dynamics and genetic recombination are the keys to introgression rates. For instance, the barrier B to introgression of a neutral allele from one parental population, across the hybrid zone, to the other can be expressed as

$$B \propto \left\{ \frac{\overline{W}_e}{\overline{W}_c} \right\}^{1/\bar{r}}$$

in which \overline{W}_e and \overline{W}_c denote the mean absolute fitness of individuals at the edges (pure parental populations) and center (mixture including hybrids), respectively, and \bar{r} is the harmonic mean recombination rate between the allele of interest and the alleles at loci responsible for low hybrid fitness (Barton, 1986). Looking at this proportionality, the lower the mean absolute fitness in the center of the hybrid

zone, the less likely that a neutral allele will traverse the zone over a given number of generations. The demographic connection is clear. The mean absolute fitness in a population is equivalent in most instances to population growth rate (Rice, 2004), and so when the hybrid zone is a population sink, the ratio $\overline{W}_e / \overline{W}_c$ is large (Barton, 1986). The demographic barrier is exacerbated by low recombination. Imagine that mean fitness in the middle of the hybrid zone is 25% lower than at the edges. A harmonic mean recombination rate of 0.5 (all are physically unlinked) will present a barrier to introgression that is only about half as strong as that presented by a recombination rate of 0.25, and only one tenth as strong as the barrier presented by recombination of 0.1. Density-dependence in population growth also affects the strength of the introgression barrier, with B increasing by the factor $(K_e/K_c)^2$ (where K_e is the carrying capacity at the edge and K_c is the carrying capacity at the centre). This makes intuitive sense, since low population size in the hybrid zone centre reduces the number of opportunities for pure-bred migrants (read alien pollen grains) to mate with hybrids. An important result of hybrid zone theory with regard to transgene introgression is that low hybrid fertility is not an absolute barrier to gene flow. It is especially important to remember that selectively advantageous alleles can cross the barrier faster than neutral ones (Barton & Hewitt, 1985).

Two wild sunflower species, *Helianthus annuus* and *Helianthus petiolaris*, illustrate the point that the ultimate level of introgression is not necessarily predicted from fertility of early generation hybrids (Heiser, 1947). Pollen fertility in the F_1 hybrids between these species ranges from 0 to 30%, with a mean of 14%. When the F_1 were crossed to each other and to the parents in order to produce F_2 and backcross progenies, seed set was 2% or lower. Yet, these two species have given rise to hybrid species in at least three locations (Rieseberg, 1991). Although the low fertility of the early generation hybrids act as a bottleneck to introgression, these low-fitness individuals can nevertheless serve as a bridge to the origin of later-generation hybrids with novel gene combinations that confer high fitness.

Neutral alleles seem to be more likely to introgress between closely related parental taxa than those more distantly related. Rieseberg and Wendel (1993) reviewed 165 proposed cases of introgression that were investigated with the use of putatively neutral molecular markers. Of the 65 studies with strong supporting evidence for gene transfer between plant taxa, two-thirds concerned introgression between races or subspecies of the same species. It makes intuitive sense that more closely related taxa would be more likely to exchange genes; the longer two taxa have been reproductively isolated, the more mutations and chromosomal rearrangements they are expected to accumulate. These genetic changes can then give rise to the incompatibilities that reduce hybrid fertility.

The specifics of chromosome rearrangements can strongly influence the chance of introgression for any given allele. This is because rearrangements alter the rate of recombination. This is clearly the case in hybrid zones between wild populations of *H. annuus* and *H. petiolaris*. Rieseberg *et al.* (1999) examined the frequency of 88 diagnostic RAPD markers located in 17 linkage groups in three replicate

hybrid zones in the central United States. All but 4 of the 139 plants collected and tested from the hybrid zone showed patterns of mixed ancestry. However, the movement of genes was not uniform across the genome. An examination of linkage maps indicated that neutral markers in the seven linkage groups that were colinear between the two parent species were nearly twice as likely to introgress as those in the 10 rearranged linkages. Some chromosomal blocks introgressed much less than random expectation, and did so to a similar degree across the three replicate hybrid zones. A smaller number of blocks apparently introgressed at a rate that was significantly higher than random expectation. This strongly suggested negative and positive selection, respectively, on these linkage groups. Further tests showed that these blocks were associated with pollen fertility. Selection during the gametic stage of the life cycle is evident in the observation that alleles frequently do not segregate in Mendelian ratios in hybrid crosses (Rieseberg & Carney, 1998).

Another important consideration is that mating between hybrids and parental types is unlikely to be random. Loss of self-incompatibility is common in crop species and so hybrids could have higher selfing rates than self-incompatible wild relatives, giving them a transmission advantage in terms of assurance of seed set. Conversely, the rate of elimination of crop alleles will be lower in progenies from hybrids that are selfed compared with those that are backcrossed to the wild parent. Differences in flowering phenology will also affect mating frequencies (Cruzan & Arnold, 1994; Weis & Kossler, 2004).

As a final point, some argue that hybridization and concurrent genome restructuring can generate novel, fertile genotypes (Levin, 1983; Mikkelsen *et al.*, 1996; Soltis & Soltis, 1999). By increasing genetic diversity, hybridization may thereby release these individuals from the genetic constraints that prevented adaptation to novel natural habitats. Although adaptation does not ensure subsequent invasion, the hypothesis of hybridization as an invasiveness catalyzer is a troubling possibility (Ellstrand & Schirebeck, 2000). It should be remembered, however, that this property is a feature of hybridization *per se* and so does not apply specifically to GM hybrids. Nevertheless, it may well be that in some cases the presence of a transgene may exacerbate this propensity towards increased invasiveness.

To summarize, the genetic complications presented by the biology of hybridization can present a number of hurdles that will interfere with the introgression of a transgene from a GM crop to wild species. Ironically, crop breeders for years have faced these hurdles while moving genes in the opposite direction – from wild relatives to crop (Rieseberg & Carney, 1998). Controlled crossing programmes and artificial selection are often successful in capturing desirable traits for crop improvement. From a risk-assessment viewpoint, we want to know how spontaneous mating patterns, coupled with natural selection, will affect the introgression rate. In other words, we would like to make quantitative statements about risk exposure for particular systems (Wilkinson *et al.*, 2003b; Poppy, 2004). The quality and nature of data we assemble relating to the ecological fitness of crop–wild hybrids is clearly vital in determining the level of certainty that we are able to reach in any semi-quantitative predictions on their subsequent behaviour.

6.2.2 Crop–wild hybridization: two examples

Ellstrand (2003) lists 48 crop species for which there is evidence of spontaneous hybridization with wild relatives, including food staples such as rice, maize, beans and wheat. In some instances the hybridizing relatives are feral populations of the crop. In others the relatives are natural populations of the same species, as with sugar beet, *Beta vulgaris* (Bartsch *et al.*, 1999). Interspecific hybrids between crops and congeneric wild species are also known, as with squash and wild gourd (*Curbubita pepo* and *Curbubita texanum*; Spencer & Snow, 2001). Some hybridization events cross generic boundaries, as in the case of wheat and jointed goatgrass (*Triticum aestivum* and *Aegilops cylindrica*; Wang *et al.*, 2001). This section examines experimental studies of crop–wild hybridization and introgression in order to indicate the types of data we have so far accumulated. The focus is on the degree to which the ‘genetic baggage’ does or does not present a barrier to introgression generally. I will compare and contrast two crop systems, sunflower and oilseed rape, that have received extensive scrutiny. A later section will ask what predictions on the introgression of transgenes can be made based on such information.

6.2.2.1 Sunflower

Sunflower, *H. annuus* var. *macrocarpus*, is cultivated over a wide expanse of central North America within the natural geographic range of its progenitor *H. annuus* var. *annuus*. Over 75% of cultivated fields are located within 100 m of wild sunflower stands, and in most cases the flowering periods of the wild and cultivated stands overlap (Burke *et al.*, 2002). This offers ample opportunity for crop–wild hybridization. The wild sunflower is strongly branched with many flowering heads. Plant breeding has created cultivars with drastically reduced branching and increased inflorescence and seed size.

Snow *et al.* (1998) evaluated several fitness components in hybrids by crossing wild materials collected from Texas, Kansas and North Dakota to each of two different sunflower cultivars. Seeds produced by hybrid crosses were generally larger and showed lower dormancy rates compared to those from wild \times wild crosses. Juvenile survival was equal between wild and hybrid plants. At maturity, the hybrids produced many fewer branches and flower heads, although for most crosses the hybrids produced larger flower heads than wild-type plants. The larger heads did not compensate for their lower numbers – and so wild plants held an advantage in seed production that ranged from around 24% for the North Dakota wild population to over 500% for the Texas population. The hybrid’s disadvantage is exacerbated by increased susceptibility to insects that feed on the developing seeds. Cummings *et al.* (1999) found that coleopteran and lepidopteran larvae destroyed 36% of the seeds on hybrids, but less than 2% on wild plants. Post-dispersal seed predation is also greater for hybrid-produced seed (Alexander *et al.*, 2001). Rodents removed 42% of wild-produced seeds from field plots, but 62% of hybrid seeds. This difference was attributed to seed size; a large proportion of wild seeds were below the rodents’ acceptable size threshold, while nearly all hybrid seeds were above it.

Despite the hybrid disadvantage, neutral crop genes flowing into sunflower populations persist. Whitton *et al.* (1997) induced a single gene flow event into a California wild sunflower population that grew adjacent to an agricultural field that had no previous history of sunflower production. The field was sown with a cultivar for one year only, and spontaneous hybridization occurred. In subsequent years wild plants were sampled and examined for crop-specific RAPD markers. In the first year following gene flow, crop-specific allele frequencies were about 0.11 in wild plants growing 3 m from the field margin, but less than 0.02 in plants growing 400 m distant, suggesting that bees moved most crop pollen only a short distance. Averaged across the entire recipient population, crop allele frequency was about 8%. The wild population was subsequently re-sampled 3 and 5 years after gene flow. During the intervening generations, the crop alleles had become more evenly spread throughout the wild population but retained their 8% overall frequency.

Given these experimental results on hybridization and the proximity of natural populations to cultivated fields, one would expect the introgression of crop alleles into the wild sunflower populations to be common. This was confirmed in a particularly rigorous study by Linder *et al.* (1998). They looked for 18 crop-specific neutral genetic markers (RAPDs) in wild plants collected from three areas in the northern Great Plains of the United States and Canada where sunflowers had been in cultivation for 20 years or more. Note that this is the same region where Snow *et al.* (1998) found the least reduction in F_1 fitness. Plants with obvious F_1 morphology were avoided, and so their collection should reflect the longer term consequences of hybridization. Remarkably, all 115 plants in the sample had at least one crop-specific marker and some as many as 14. Average frequencies of the marker alleles ranged between 0.3 and 0.4 in the wild populations, which were orders of magnitude higher than in wild populations outside the sunflower production region. It could be argued that the similarity among populations could be due to convergent evolution; however, restriction fragment analysis of the RAPD markers demonstrated their homology. Finally, markers that were physically linked in the crop tended to be linked when they occurred in the wild population, and this indicates that introgression has been recent (insufficient time for recombination to restore linkage equilibrium). One is left to conclude that transgenes of neutral or positive selective value would move easily into wild sunflower populations in this region despite the lower fitness of early generation hybrids.

6.2.2.2 *Oilseed rape*

Oilseed rape is cultivated worldwide and co-occurs with one or more sexually compatible wild relative in nearly all locations. Volunteer oilseed rape can itself be a weed when spilled seed germinates after rotation to another crop species. Seed spilled during transport can establish successional feral populations at field margins and along roadsides (Crawley & Brown, 1995). It is reasonable to expect minor or no barriers to hybridization between crop and feral populations.

Evaluating the potential for introgression from *Brassica napus* into wild populations of the congener *Brassica rapa* involves some additional genetic complexities. Oilseed rape hybridizes with both weedy (Warwick *et al.*, 2003) and naturalized

ecotypes of turnip rape, *B. rapa* (Wilkinson *et al.*, 2003a). The success of crosses between these two species is not surprising, given their shared ancestry: *B. napus* is an allopolyploid derived from hybridization of *B. rapa* with *B. oleracea*. Genomes of the diploid parental species are designated AA and CC, respectively, so that the *B. napus* genome is AACC (U, 1935). When *B. rapa* is crossed to *B. napus*, the offspring are AAC, i.e. triploid. Extensive co-linearity of the A genomes in the two species probably facilitates introgression.

There has been considerable experimental work on introgression from *B. napus* to *B. rapa*. Both weedy and natural populations of the latter occur in proximity to the fields of the former over most of the United Kingdom, where Wilkinson *et al.* (2003a) estimate that tens of thousands of hybrids can be formed every year. Although success of this interspecific cross (seeds per fruit) is lower than crosses within the parental species, survival of the resulting hybrid seedlings is equal to that of *B. rapa*, and plant size and flower production is greater (Hauser *et al.*, 1998b). Reports vary on F₁ reproductive success, with some studies (Hauser *et al.*, 1998b; Vacher *et al.*, 2004) showing higher seed production than *B. rapa*, and others (Hauser *et al.*, 1998a) showing lower. The reproductive success of F₁ through seed production can change with their frequency in the population; when rare compared to the wild parent, they are more fertile (Hauser *et al.*, 2003), suggesting that they are more successful as mother when they are likely to receive wild pollen. Similarly, when wild-hybrid populations are grown at high density, F₁ reproductive success through seed increases, both through increased pollination by *B. rapa* and by the competitive advantage from hybrid vigor for vegetative growth (Vacher *et al.*, 2004). However, the F₂ and backcross generations suffer reduction in most fitness components, including seed set and pollen fertility (Hauser *et al.*, 1998a). When these components were combined, the fitness of the F₂ generation was only 15% as great as *B. rapa*, and the backcross only 17% as great. However, individuals within these later-generation hybrids were not uniformly unfit. The original crosses drew parents from two *B. rapa* populations and two *B. napus* cultivars. Both population and cultivar affected fitness, as did variation among families within population and cultivar. Some wild population-cultivar combinations produced a few F₂ plants, with an overall fitness well above the *B. rapa* average. The message is that sufficient underlying genetic variation exists in the parental species to generate some moderately fit recombinants in later generations, which then can serve as a bridge for introgression from crop to wild population.

The breakdown in mean fitness of the F₂ generation hybrids and backcrosses, as well as the higher fitness variance, can be traced in large part to their aneuploidy. An F₁ hybrid (AAC) will produce gametes with one copy each of the 10 A genome chromosomes (absent crossing over, an average of 5 from the maternal and 5 from the paternal parent). However, each of the 9 C genome chromosomes (all from the *B. napus* parent) has only a 50% chance of being passed to any given gamete. Thus, chromosome numbers in the F₁ × *B. rapa* backcross range from 20 (AA) to 29 (AAC). Lu *et al.* (2002) have shown that chromosome number approximately follows a binomial distribution, with the mode at 24.5 (Figure 6.1). If fitness is independent of chromosome number, there is a 50% chance of loss for each C

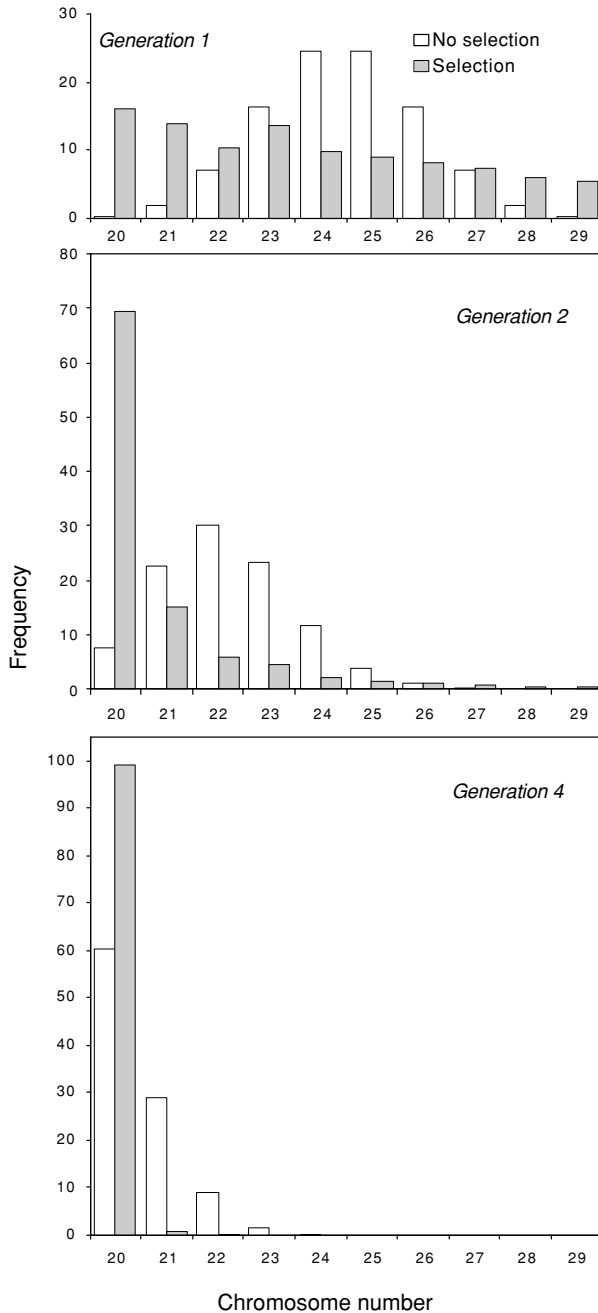


Figure 6.1 Distribution of chromosome number in progressive generations of backcrossing of *B. napus* × *B. rapa* hybrids to the *B. rapa* parent. Offspring receive a full haploid complement of A genome chromosomes from each parent, but each C genome chromosome has only a 50% chance of transmission from the hybrid parent. If fitness were independent of chromosome number, the representation of the C genome would be cut by half every generation. However, intermediate chromosome numbers confer low fitness (see Figure 6.2), and so the C genome is virtually eliminated by backcross generation 4. Data from Lu *et al.* (2002).

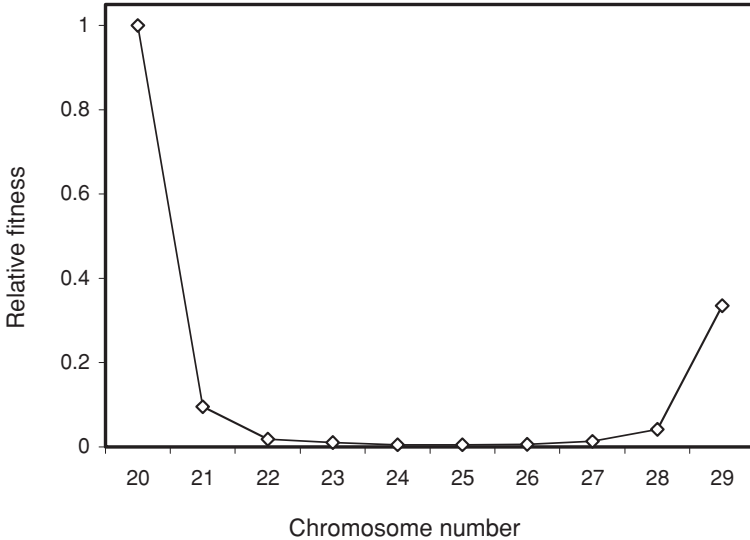


Figure 6.2 Average fitness as a function of chromosome number in $F_1 \times B. rapa$ backcrosses. Data reported in Lu *et al.* (2002).

chromosome within a lineage in each backcross generation. Thus, genes in the C genome do not segregate in Mendelian ratios. By the BC_4 generation, enough chromosomes are lost by random segregation that over 60% of the population is expected to revert to the *B. rapa* configuration of 20 A chromosomes (Figure 6.1). Further, Lu *et al.* showed there is disruptive selection on chromosome number in hybrids; the relative fitness for $2n = 20$ is 1.00, and 0.33 for $2n = 29$, but much lower for intermediate chromosome numbers (Figure 6.2). With continued backcrossing and selection, the individuals with the higher fitness $2n = 29$ configuration (AAC) disappear by random segregation and the lower fitness intermediate configurations are eliminated by both segregation and selection. With the observed fitness function (Figure 6.2), Lu *et al.* predicted that by the BC_4 generation (Figure 6.1) the population is virtually transformed back to the *B. rapa* configuration, $2n = 20$ (AA). By the same logic, chromosome numbers for F_2 offspring could range from $2n = 20$ (i.e. the AA configuration of *B. rapa*) to $2n = 38$ (the AACCC configuration of *B. napus*).

What would happen in a *B. napus*–*B. rapa* hybrid zone? One can imagine that with continued bi-direction gene flow from crop and wild populations, plants in the zone would come to an equilibrium distribution of chromosome numbers. The two parental configurations would predominate, while intermediate configurations settle to lower frequencies determined by the balance between the rate at which they are formed by mating between unlike individuals and their elimination rate, caused by selection and recombination. Of course, with crop rotation and other management

practices, the idealized hybrid zones depicted in conventional population genetic theory are highly unlikely. Nevertheless, existence of areas where the two parent species and their hybrids persist, at least in the mid-term, has been seen.

Introgression between *B. napus* and *B. rapa* was demonstrated by Hansen *et al.* (2001), who examined a mixed population that occupied a field that had been under organic cultivation for 11 years. Both parental species had been free to spontaneously hybridize over that time. AFLP markers deemed to be diagnostic of the crop and *B. rapa* were examined in 102 plants from the population. One of the plants was found to be an F₁ hybrid (possessing all markers from both parental species), 44 were judged to be introgressed (had some from each parental species) and the remainder appeared to be either one or the other of the 2 parental species. Most of the introgressed individuals had all 3 of the *B. rapa*-specific markers plus 1 or 2 of the 21 *B. napus* markers (most of which were known to occur on the C genome). This distribution of *B. napus* markers in the plants that also had *B. rapa* markers could be used to get a rough idea of how far introgression had proceeded. Hansen *et al.* compared the observed frequency distribution of *B. napus* markers among plants that also had *B. rapa* ancestry (i.e. first to advanced generation backcrosses) to the distribution seen in BC₁ and BC₂ generations of controlled crosses. The natural population more closely matched the BC₂. The simplest interpretation of these patterns is that although successful matings between parental species are infrequent (F₁s are rare), the few successful hybrids can give rise to lineages that continue to backcross to the wild relative. Although marker frequency distributions resembled those of the BC₂ generation, the number of markers examined was insufficient to get a precise picture of the true degree of introgression. There are 19 chromosomes in the haploid genome of *B. napus*, and so with only 21 markers, most of the intergenomic recombinants (crossovers in collinear regions of A and C chromosomes) would go undetected, thus underestimating the number of introgressed individuals.

6.3 Projecting introgression of transgenes using empirical data and models

The logical first steps in assessing the risk of transgene introgression from crop to wild populations are to evaluate the rate at which pollen or seeds migrate into the receiving population, and to examine fitness components in the hybrids descending from them. However, even the most elegant data sets cannot by themselves yield a statement of likelihood for the particular path that a transgene takes in the introgression process. A theoretical framework based in population genetics and ecology is needed. Unfortunately, significant hurdles need to be cleared before a comprehensive and general framework can be constructed. A full resolution of the transgene problem requires a synthesis of two fields from population genetics: hybrid zone theory and the demographic approach to natural selection. The first is important because the fate of an escaped transgene depends not only on its fitness effects on individual hybrid plants in a wild habitat but also on the fitness effects of all the

crop alleles that come with it in gametic disequilibrium and linkage disequilibrium, and the rate at which reassortment and recombination restores equilibrium. These effects are probably idiosyncratic to each crop–wild system and may vary between geographic regions, as suggested by the sunflower and oilseed rape examples. To be of use for risk assessment, any model framework used to analyse these initial stages of gene transfer would have to accommodate the particulars of individual cases. This renders the classical theories on hybrid zones, which describe systems of bi-directional gene flow that have come to equilibrium, unsuitable for our purposes. Other theoretical models have given general ideas on what factors facilitate or impede introgression, but these are not applicable to real-world cases because they invariably pool all hybrid types into a single category in order to be mathematically tractable (e.g. Haygood *et al.*, 2003; Thompson *et al.*, 2003).

Demographic genetics, the second ingredient for the needed synthesis, is especially valuable in assessing the ecological impacts of transgene introgression. It rests on ideas discussed by Dobzhansky (1968) and others (e.g. Mueller *et al.*, 1991; Charlesworth, 1994; Anderson & Watanabe, 1997), specifically, that demographic parameters such as the finite rate of increase (λ) or the intrinsic rate of increase (r) can be attributed to genotypes as meaningful measures of their fitness. Suppose, for instance, a newborn individual with genotype *AA* is expected to contribute λ_{AA} offspring to the next generation. Likewise, *Aa* and *aa* genotypes have expectations of λ_{Aa} and λ_{aa} , respectively. Further suppose that *A* is a new allele introduced by gene flow into a population with a stable size, i.e. $\lambda = \lambda_{aa} = 1$. If $\lambda_{AA} \geq \lambda_{Aa} > \lambda_{aa}$ (or, if $\lambda_{AA} > \lambda_{Aa} \geq \lambda_{aa}$), then once the new gene is introduced, the overall λ for the population will be greater than 1, and not only will the *A* allele spread but the population will grow. As a note of clarification, λ_{ij} is the expected number of offspring that an *ij* produces, which is not the same as the rate of increase for the *ij* genotype (Denniston, 1978); for instance, the rate of increase for the *AA* genotype will depend on the λ_{ij} for all genotypes that can produce *AA* offspring, and that includes *Aa* parents. Clearly, the demographic genetic approach can assess both the genetic and population consequences of transgene introgression into a receiving population. One can ask how a change in a specific modified trait changes fitness components and thereby changes λ and then projects the consequences. Muir and Howard (1999, 2001, 2002) have used just such an approach in their net-fitness model that explored the potential effect of a genetic modification for growth hormone production on a receiving wild fish population.

To apply this approach, one must first estimate the λ_{ij} and be able to give some expression of their confidence in these estimates. The estimates will in large part come from fitness component measurements, like female fecundity, germination rate, seedling survivorship, etc. These quantities are often measured without great difficulty. Other components of fitness, such as male reproductive success, are notoriously difficult to estimate. Here I will present several cases where modelling frameworks have been integrated with empirical data to give insights into fitness and its components. As I proceed, I will comment on their value in assessing potential ecological impacts of transgene introgression.

6.3.1 How well do fitness estimates predict population change?

As the saying goes, the proof is in the pudding. Some of the experiments I have described above sought to measure fitness components in crop–wild hybrids and compare them to wild populations. The other studies have asked, after the fact, how much introgression occurred in populations where introgression was expected. Ideally, we would like a framework that takes information from the first type of study to predict the outcome of the second.

One approach follows standard one-locus, two-allele theory to make short-term predictions on the rate of increase or decrease of introgressed individuals. Cummings *et al.* (2002) used this approach to predict a one-generation change in marker genotype frequencies in mixed wild-hybrid sunflower populations. They created replicated synthetic populations with equal numbers of wild and crop–wild F₁ hybrid plants that expressed distinguishing isozyme markers. This scenario resembles the immediate aftermath of a major gene flow event, as would be seen after a sunflower crop is grown adjacent to a wild sunflower population for a single rotation. They then collected progeny and determined their genotype frequencies. Whereas Cummins *et al.* looked at neutral markers, I discuss their approach as it could be applied to transgene introgression.

First, define the frequency of the four possible genotypes as P_{00} , P_{01} , P_{10} and P_{11} , where subscript 0 denotes lack of the transgene (the wild-type) and 1 its presence. Frequencies of the two types of hemizygotes are noted separately (P_{01} and P_{10}) in order to distinguish individuals receiving the gene through their mother from those receiving it through their father. The reason for doing so will become apparent.

Assume that the genotypes have pollen viability m_{ij} and seed fecundity f_{ij} , scaled so that mean viability and fecundity are both 1, and that seed-to-adult survivorship is denoted by w_{ij} . Also assume that the experiment includes only F₁s and wild types as parent so that only ‘00’ and ‘01’ genotypes are present in the initial generation. With random mating, the four genotype frequencies among the adult plants in the offspring generation are expected to be

$$\begin{aligned}
 P_{00}' &= \frac{w_{00}}{\bar{w}} \left[P_{00} f_{00} \left(P_{00} m_{00} + \frac{1}{2} P_{01} m_{01} \right) + \frac{1}{2} P_{01} f_{01} \left(P_{00} m_{00} + \frac{1}{2} P_{01} m_{01} \right) \right] \\
 P_{01}' &= \frac{w_{01}}{\bar{w}} \left[P_{00} f_{00} \left(\frac{1}{2} P_{01} m_{01} \right) + \frac{1}{2} P_{01} f_{01} \left(\frac{1}{2} P_{01} m_{01} \right) \right] \\
 P_{10}' &= \frac{w_{10}}{\bar{w}} \left[\frac{1}{2} P_{01} f_{01} (P_{01} m_{01}) + \frac{1}{2} P_{01} f_{01} \left(\frac{1}{2} P_{01} m_{01} \right) \right] \\
 P_{11}' &= \frac{w_{11}}{\bar{w}} \left[\frac{1}{2} P_{01} f_{01} \left(\frac{1}{2} P_{01} m_{01} \right) \right]
 \end{aligned}$$

in which w_{ij}/\bar{w} normalizes the frequencies after juvenile mortality so that they sum to 1. Within the square brackets we see several terms that depict the frequencies

of the various matings that can produce the specified offspring genotype. Take for instance the last of the four equations, which gives expected P_{11}' , that is, the expected frequency of homozygotes for the transgene in the offspring generation. Given that only '00' and '01' individuals were present in the parental generation, '11' offspring can only be produced by a mating between two '01' parents, that is, two F_1 hybrids. The frequency of ovules with the '1' allele is equal to the frequency of '01' mothers (P_{01}) \times the relative number of ovules these mothers produce (f_{01}) \times the fraction of their ovules that receive the '1' allele (1/2). Similarly, the frequency of '1' pollen grains is $\frac{1}{2}P_{01}m_{01}$. The probability that these two gamete types combine is the product of their frequencies (assuming random mating). In contrast, null homozygotes, '00', can be produced by the union of two wild types, a wild mother and hybrid father, a hybrid mother and wild father or by two hybrids. Hence there are more terms in the first equation than the last. This particular set of equations represents the combination of parental genotypes tested by Cummings *et al.*, but other sets are appropriate for other combinations (e.g. Prout, 1971; Winterer & Weis, 2004).

Cummings *et al.* (2002) used the model prospectively, that is, they inserted their estimates of the fitness components into the model to see if they could predict genotype frequencies in the offspring generation. They started with equal proportions of wild and hybrid parents and so the initial frequencies of the crop alleles were 0.25. As expected from previous studies, the fecundity of F_1 plants was significantly lower than wild type (fewer flower heads). This fecundity difference was incorporated into a model to predict frequencies of the three markers in the offspring generation. Since experimental conditions led to nearly 100% survivorship, all w_{ij} were assumed to be 1.0. In addition, because of the difficulty in measuring pollen production and viability, it was assumed that both wild and F_1 parents were equal. If fecundity were equal between the two parental genotypes (both $f_{ij} = 1$), offspring genotypes would have followed Hardy-Weinberg proportions. This was not the case. However, when the measured fecundity differences were entered into the model, the observed offspring genotype ratios matched the prediction quite well. Specifically, the frequency of the crop allele was 0.25 in the parental generation, but fell to 0.05 among the offspring, as predicted from fecundity differences. Levels of pre-dispersal seed predation by lepidopteran and coleopteran larvae were added to the model, but this did not improve the fit. They also tried to account for assortative mating (hybrids flower earlier than wild type) but this did not improve the fit. However, they did not have access to new methods for prospective estimates of assortative mating (Vacher *et al.*, 2004; Weis & Kossler, 2004). A re-analysis with these new estimation methods might give a different answer.

Cummings *et al.* (2002) certainly took a step forward in quantifying risk during the early stages of introgression. However, their work also reveals some limitations in the prospective method. For instance, there is the difficulty in measuring reproductive success through male function, m_{ij} . Apparently there are no differences in this case (or perhaps the differences for different mating combinations are offsetting) but this will not always be so (e.g. Pertl *et al.*, 2002).

Fortunately, a model like this can be used in two ways – prospectively or retrospectively. In the prospective approach, used by Cummings *et al.*, estimates of m_{ij} , f_{ij} and w_{ij} can be obtained in a greenhouse or microcosm experiment, and then inserted into the equations to make predictions on the outcomes of further experiments, such as those done under field conditions. If the outcome and prediction do not match, investigators can explore the reasons for failure, which might include sensitivity of fitness components to the environment or the violation of assumptions such as random mating. However, the same models can be used retrospectively (Prout, 1971). That is, put parents of known genotype into experimental populations at various frequencies and allow them to reproduce. Then determine the genotype frequencies among the resulting offspring. With these two pieces of information in hand, parental and offspring genotype frequencies, values of m_{ij} , f_{ij} and w_{ij} can be estimated.

The work by Prout (1971) on estimating fitness components in *Drosophila* points to a way to estimate fitness components, and indeed total fitness, retrospectively. This approach uses the same type of experimental set-up as Cummings *et al.*. Using the equations above, one can make a maximum likelihood estimate of female and male fitness components using only the starting parental frequencies and the observed offspring genotype frequencies. The approach works because this set-up is a one-generation selection experiment. By finding the amount of evolution that occurred over that generational transition, one can back-calculate the amount of selection that must have produced it. In fact, plants are in some ways more amenable to this analysis than *Drosophila*. Plants hold on to their offspring before dispersal and this allows one to collect a sample of offspring for whom the genotype of the mother can be known with certainty. This then makes it possible to distinguish the two types of heterozygotes: those receiving the transgene from the mother and those receiving it from the father. (Separate experiments are required to do this in species that scatter their offspring.) Distinguishing between the two heterozygotes expands the model from three to four equations, and with four equations, three parameters can be estimated. This opens the possibility of measuring things like differential genetic compatibility – is F_1 pollen more successful on F_1 stigmas or wild stigmas? – or of retrospectively estimating assortative mating among the parents (see Winterer and Weis, 2004, for appropriate equations). In addition, it can more thoroughly explore density-dependent fecundity and frequency-dependent mating success such as seen with *B. napus*–*B. rapa* hybrids (Pertl *et al.*, 2002; Hauser *et al.*, 2003; Vacher *et al.*, 2004) and decompose success into its female and male components.

The prospective and retrospective estimation approaches can be combined when needed. For instance, easy fitness components like seedling survivorship could be measured directly and the difficult ones retrospectively. One can then determine which fitness components are responsible for the total genotypic variation in fitness. This can be done by inserting the prospectively estimated components into the model equations as constants, one by one and in combinations, and then estimate the remaining components retrospectively by maximum likelihood.

Various model selection methods, such as the Akaike Information Criterion or Schwartz's criterion can then be used to determine which combination of components gives the best fit with the fewest parameters (Johnson & Omland, 2004). This is important because genotypes could show large differences for one fitness component, and yet hardly differ at all for total fitness (Bergelson, 1994; Muir & Howard, 1999).

The prospective/retrospective method for estimating fitness components will be useful if the results can be incorporated into a demographic projection. For instance, these projections on the dynamics of early generation hybrids inform decisions on the need for long-term monitoring. However, there is still a glaring problem – the lack of an existing framework that will incorporate the fitness effects of crop genes entering the wild population in gametic disequilibrium and linkage disequilibrium. In the model presented above, the fitness effects of the transgenes are not separated from the fitness effects exerted by the crop genes it brings along in gametic disequilibrium. For gametic disequilibrium, where insertion site is of no importance, one can perform single-generation selection experiments that pit wild genotypes against F_1 , F_2 or BC_n genotypes carrying the transgene, or pit these hybrids one against the other. The trick will be erecting a framework that will be able to take these data and put them into a demographic model that will predict the time course of introgression, and do so under various assumptions about the temporal pattern and intensity of continued gene flow from the crop, and gene flow from the hybrid zone near the field margin to more distant wild populations. In summary, there is much to be done before we have a modelling framework that can predict the flow of a transgene out of the fields, through hybrids, and into the wild genetic background. And so, there is not yet any reason to dissent from the skepticism of Kareiva *et al.* (1996) that the combination of experiments and models will allow robust risk assessment, at least with respect to the early stages of introgression. It will be more problematic to accommodate for linkage disequilibrium, and this will require detailed information on recombination rates between the transgene and negatively selected crop alleles.

6.3.2 *A way around the difficulties?*

A little more than a decade ago, when transgenic plants were first introduced, there was a concern in the general public that genetically modified crops *per se* posed an environmental risk. It was feared that somehow modified crops, by virtue of the genetic transformation process alone, could become super weeds. Early and extensive experiments by Crawley *et al.* (1993) did much to dispel this notion by showing that oilseed rape modified for herbicide tolerance or kanamycin resistance is no more capable of sustained population growth outside cultivated habitats than is conventional oilseed rape. Of course, there was no reason to suspect that either of these two traits would be advantageous – natural habitats are not regularly inundated by herbicide or antibiotics. Nevertheless, this work is of lasting importance because it put the focus on the minimal condition for a GM crop to be any more invasive than

its conventional counterpart, namely that the finite rate of increase of the former had to be greater than the latter in the appropriate habitat.

Do other traits, such as insect and disease resistance, have the potential to increase fitness in wild recipients once the gene recombines into the wild genetic background? The one published study to date to test for such effects in an ecologically relevant environment indicates that this can be the case. Pilson *et al.* (2004) compared fecundity of BC₁ and BC₃ generations of *H. annuus* carrying a Bt transgene for resistance to Lepidoptera to non-transgenic controls. By the BC₃ only 12.5% of the crop genome remains, assuming free recombination, and so while the transgene was not totally introgressed into the wild genetic background, it is more informative than earlier generation hybrids. Damage to the developing seed was essentially reduced to zero in the Bt plants, and this increased seed production by 15–55%, depending on the year and field site. This is not a surprising result since previous work showed that pre-dispersal seed predation is a potent selective force on *H. annuus* (Pilson, 2000).

But the next question is whether adaptive transgenes change the population dynamics of the species. A given increase in fecundity may have a trivial effect on population growth rate if the population is limited by seedling recruitment (Bergelson, 1994). How does one know? Several groups working on transgenic risk assessment have taken the important step of adopting methods employed in conservation biology and biological control. Researchers in these areas use matrix projection models (Caswell, 2001) to estimate the short- to mid-term effect of management practices on target populations. This method estimates the change in population growth that could be achieved by increasing or decreasing one or more fitness components (e.g. McEvoy & Coombs, 1999; Parker, 2000; Ehrlén, 2003).

The explanation for matrix projection best starts with a life cycle diagram. These diagrams can be complex, as I will show later, but a simple one will explain the method. Consider an annual plant in a uniform environment (Figure 6.3). Each year a seed sitting in the seed bank has some probability g of germinating and growing into a reproductive adult. It also has a probability b of surviving in dormancy in the seed bank for the next year. Of those that germinate and reach adulthood, they will on average contribute r new seeds to the seed bank. These life-stage transitions can

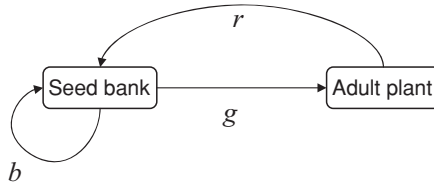


Figure 6.3 Life cycle diagram for a hypothetical annual plant. The term g denotes the probability that a seed germinates and survives to adulthood, b is the probability that it stays in the seed bank and survives to the next year and r is the average number of seeds an adult contributes to the seed bank.

be placed in a matrix

$$A = \begin{bmatrix} b & r \\ g & 0 \end{bmatrix}$$

The first row of the matrix denotes the number of seeds in the seed bank at the end of the season contributed by those that do not germinate (who contribute by not dying) and those that reach adulthood (who contribute by reproduction). The second row denotes the probability of transitioning from the first life stage to the next; this is g for germinating seeds making it to adulthood and zero for adults. The matrix is always square, and its dimension is equal to the number of life stages examined. The matrix entries are products of one or more fitness components: for example, g is the probability of germination \times seedling survivorship \times juvenile survivorship; r is the daily survival rate \times daily seed production, summed over all the days in the reproductive period. This matrix can then project the population size for each life stage, and hence for the whole population, in the next generation by the equation

$$\begin{bmatrix} n_{s \ t+1} \\ n_{a \ t+1} \end{bmatrix} = \begin{bmatrix} b & r \\ g & 0 \end{bmatrix} \times \begin{bmatrix} n_{s \ t} \\ n_{a \ t} \end{bmatrix}$$

where subscripts s and a stand for seed and adult, respectively. This equation can also be written in matrix notation as

$$\mathbf{n}_{t+1} = \mathbf{A} \times \mathbf{n}_t$$

where \mathbf{n}_t is the vector containing the number of individuals at each life stage at reproduction during the current season, and \mathbf{n}_{t+1} the vector for the number there will be at the end of the next. The finite rate of increase for the population, λ , is the dominant eigenvalue of \mathbf{A} . Total population size can be projected x generations into the future by the equation

$$N_{t+x} = N_t \lambda^x$$

If fitness components are constant, the population comes to a stable stage distribution, and each stage increases by rate λ (Caswell, 2001). Values for the matrix entries can be derived prospectively or retrospectively under relevant conditions.

The matrix projection is useful because one can play ‘what if’ games to see how changes in one or more fitness components affect λ . This is done by measuring the elasticity of λ to the various matrix elements; for instance, if g is changed by a certain percent, what will be the percent change in λ ? As an example, Shea and Kelly (1998) wanted to determine why nodding thistle, an invasive species, was not being suppressed in New Zealand by the thistle receptacle weevil (*Rhynocyllis conicus*), which had been imported for biological control. They measured fitness components

in different thistle populations over several years and constructed composite matrices. The finite rate of increase in the absence of weevils was close to 2.0 (which underscores the thistle's invasive status). When the typical 30–40% reduction in seed set caused by the weevil was factored in, λ still exceeded 1.0. Simulations using the matrix methods showed that beetles would have to destroy over 69% of the seeds in order to cause population decline. In other words, elasticity for seed production (r in the hypothetical matrix) is low. On the other hand, analysis showed that elasticity for the seed-to-adult transition was large, and so effective control practices should concentrate on this life stage.

This modelling approach can be used to predict the effect of a transgene on the finite rate of increase in a receiving population. For instance, Deville (in press) examined the demography of feral oilseed rape growing in field margins and road verges in France. Besides reproduction *in situ*, these populations are maintained by occasional seed input directly from adjacent fields and by spillage from trucks. Land managers control these populations by herbicide application and mowing. If herbicide-resistant genes introgress into these feral populations, herbicide control would be weakened and there would be opportunity for reverse gene flow back into non-transgenic weed populations in agricultural fields. The life history diagram for these feral populations is shown in Figure 6.4. Nine life stages are represented, including seeds in the seed bank, different vegetative rosette classes for those developing from seeds that germinate in fall and those germinating in the spring, and three adult classes, denoting plants that are not mowed, those mowed once, and those mowed twice. The figure indicates the stages where mowing and herbicide application can typically occur. The effectiveness of these control measures can be evaluated by calculating fitness components in their presence and absence. Simulations were run to determine the probability of extinction within 10 years for various control combinations. Not surprisingly, herbicide application had a high elasticity. Thus, introgression of an herbicide-resistant gene would contribute significantly to population persistence (assuming the same herbicide is being used for control). In addition, they found predicted persistence was increased when some fraction of seeds stay 1 or more years in the seed bank. The fraction need not be large; even infrequent dispersal through time can be sufficient to rescue a dwindling feral population. Significantly, another trait that has been genetically modified can influence seed dormancy. Linder and Schmitt (1995) found that seeds from lines engineered for high stearate content had greater seed survival and dormancy. Thus, Deville's model suggests that this transgene could also be adaptive under natural conditions. Although Deville did not mention herbivory, her model could be easily expanded to determine whether the *Bt* gene, which reduces defoliation and thereby increases seed set in feral canola (Stewart *et al.*, 1997), would spread in these French populations.

This approach was taken to a higher level of complexity by Colbach *et al.* (2001a,b) in their GENESYS model for introgression of herbicide tolerance into feral oilseed rape. Their goal was to incorporate the effects of various management practices (crop rotation, stubble breakage, plowing methods, plant date, herbicide

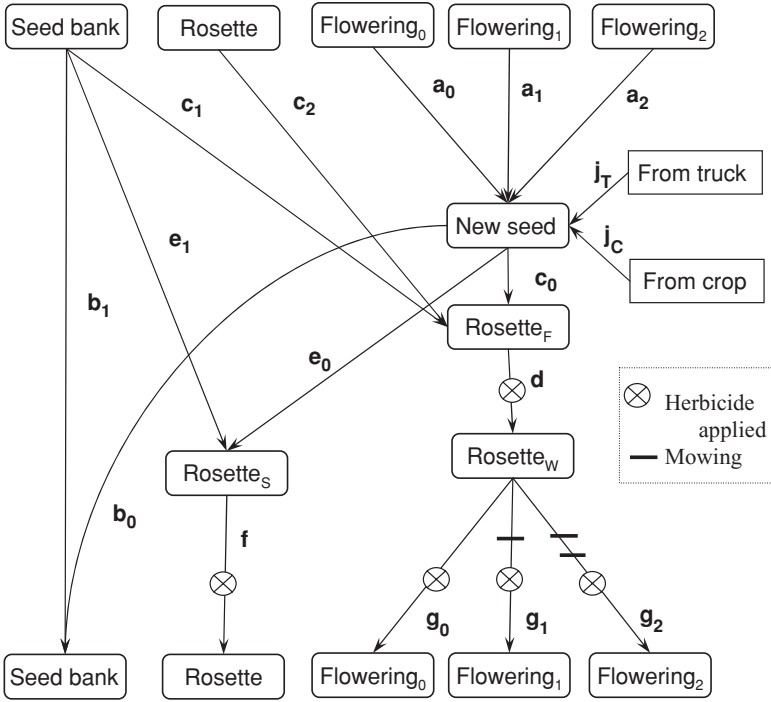


Figure 6.4 Life cycle diagram for feral populations of oilseed rape in France. Subscripts for the rosette stages are F = germinated before winter, W = survived winter and S = germinated in the spring. Subscripts for the flowering stage are 0 = not mowed, 1 = mowed once and 2 = mowed twice. Letters beside arrow are the transition parameters between life stages. Those transitions affected by herbicide application and by mowing are indicated.

applications, etc.) to rank their effect on the escape of herbicide-tolerant genes into volunteer populations. One notable advance for this model was its explicit spatial structure. Their simulations suggest that management of feral populations at the field borders and adjacent roadsides will reduce the frequency of herbicide tolerance in field volunteers.

6.4 Conclusion: a reason for hope, a cause for concern

In assessing the environmental risk posed by GM crops, Poppy (2004) has noted that ‘The weight of evidence needed to identify a possible hazard is an order of magnitude less than is needed to perform a full-scale evaluation of exposure’. It is relatively easy, for instance, to determine if Bt is toxic to caterpillars of a treasured butterfly species. It is much more difficult to determine whether a *Bt* gene escaping from a field crop into that butterfly’s host plant will spread far enough to impact adversely

the butterfly population. The demographic approach offers hope for addressing key portions of the exposure assessment. Even if we are unable to make precise quantitative predictions on the early stages of introgression, we can evaluate the potential exposure component of risk should introgression reach an advanced stage. If we find that a gene conferring insect resistance, for instance, is highly unlikely to change the finite rate of increase of the receiving wild population, then we can decide that the exposure is sufficiently low, given the hazard that commercial release of a transgenic-resistant variety is reasonable. Indeed, if we were to gather detailed demographic analysis of the important recipient wild species of all the major crops in all their areas of co-occurrence, regulators would have an important off-the-shelf tool for deciding which types of trait modification, because of their potential demographic effect, deserve special scrutiny, which are less worrisome, and which should be banned out of hand. This will shift the focus away from probability of hybridization as a decision criterion (e.g. Stewart *et al.*, 2003), which as shown earlier, does not do well at predicting whether introgression will eventually occur. Hybridization and the early stages of introgression will still remain an extremely interesting topic for study in its own right, and for the insights it may give on transgene placement in the genome (Metz *et al.*, 1997; Lu *et al.*, 2002; Stewart *et al.*, 2003). It is somewhat surprising that although the demographic approach was first suggested a decade ago by Crawley *et al.* (1993) and Kareiva *et al.* (1996), those concerned with risk assessment for transgenics have been slow to take it up, with Muir and Howard (1999, 2001, 2002) being notable exceptions.

But, the hope is tinged with concern. In cases where projections give uncertain or marginal results, the only way to verify prediction is to purposely introgress the transgene into wild genetic backgrounds and use it for multi-generation field trials. This raises an ethical dilemma: in order to make sure an action does not cause irreversible environmental harm, we must expose some environment to the risk of irreversible harm. Yet, this is not so different from risk assessment for transgenics as currently practiced, and perhaps in the long run a bit safer because decisions on what and where to test will be better informed.

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7 Assessing the environmental risks of gene flow from GM crops to wild relatives

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7.1 Introduction

Risk is a function of hazard and exposure. For the flow of transgenes from a GM crop to a wild relative to pose a risk to biodiversity, there must be both an exposure and a hazard component. That is, the transgenes must be present in populations of the wild relative (exposure) and at the same time have potential to cause a specified detrimental effect (hazard). Other chapters in this book and other recent reviews (e.g. Ellstrand, 2003; National Research Council, 2004) provide detailed analyses to evaluate how transgenes might move from crops to wild relatives and to describe the hazards that the transgenes might pose. However, data on the extent of exposure and the severity of a named hazard on their own are insufficient to judge whether the risks of gene flow are acceptable or unacceptable; in other words, the data *per se* do not constitute a risk assessment. There is inevitably need for decision making. In this chapter, we describe how hazard and exposure data can be used to assess risks of gene flow from crops to wild relatives and how decisions might be made from estimates of risk.

7.2 What is risk assessment?

Environmental risk assessment is fundamentally a tool to help make regulatory decisions; it is not necessarily a method of setting an agenda for scientific research (Hill & Sendashonga, 2003). Moreover, the risk assessment, and the decisions based on it, requires that sufficient information be made available to judge whether the environmental risks of a proposed course of action are acceptable within the context of the regulatory framework. Risk assessment itself does not seek scientific information to develop theory or even to acquire data unless their provision can improve the quality of decisions. Indeed, an efficient risk assessment should seek to minimise information to the data required to reach a sound judgement. This is because the consideration of superfluous or only marginally relevant data has a dilution effect, can confuse decision making and, equally importantly, divert effort and resources away from more worthwhile activities (Raybould, in press(a)).

The agricultural environment is in continual flux and subject to changes in management practice, subsidies, pesticide and herbicide use, cropping system and cultivar composition (e.g. autumn-sown versus spring-sown crops). In some

circumstances, therefore, it is entirely plausible that current practice, or a proposed but unregulated change, would present a greater risk to the environment than an alternative change mediated by the commercial release of a specific GM cultivar. In these circumstances, cognisance must be taken that the overall risk to the environment may be increased rather than reduced if the collection of additional data unnecessarily delays the introduction of a beneficial product (Cross, 1996).

A structure for risk assessment is vital for clarity in decision making and efficiency of data generation. A generic approach to risk assessment can be described using a small number of terms with precise meanings: assessment endpoint, hazard, exposure, an estimator of risk and a trigger value of the estimator. We give general definitions of each term and then consider how the terms apply to the risks of gene flow from crops to wild relatives.

- *Assessment endpoint.* The assessment endpoint is a precise definition of the environmental variable to be protected and should comprise an entity (e.g. a population of a particular species in a particular area) and a property of that entity (e.g. the size of the population) (Newman, 1998).
- *Hazard.* A hazard has the potential to cause some undesirable outcome; a chemical might be hazardous because it is toxic. For risk assessment, we need to estimate the magnitude of the hazard, for example the dose or concentration of the chemical required to elicit a defined effect, such as death of 50% of the population of a specified test organism (the LD₅₀ or LC₅₀, respectively) in a laboratory study. Responses to treatments in studies are called *test endpoints* and are vital for risk assessment because they provide an operational definition of the magnitude of hazard.
- *Exposure.* Exposure is a measure of the likelihood of encountering the hazard. As with the estimate of hazard, risk assessment needs an operational definition of exposure. For example, we could define exposure as the predicted environmental concentration (PEC) calculated as the maximum concentration of a chemical a specific distance away from the site of a specified pattern of use. The method of calculating exposure can also be regarded as an endpoint.
- *Estimator of risk.* Risk (R) is a function of hazard (H) and exposure (E). However, this statement is of no value if the function (estimator of risk) is not specified. For example, if the estimator of hazard (\hat{H}) is the minimum size that causes an unacceptable effect, and if the estimator of exposure (\hat{E}) is the frequency with which a hazard of that size or greater is met, then the estimator of risk could be $\hat{R} = \hat{H} \times \hat{E}$. Conversely, if the hazard estimator is the LC₅₀ and the exposure estimator the PEC, then suitable estimators of risk are $\hat{R} = \hat{H} / \hat{E}$, the toxicity exposure ratio (TER), or $\hat{R} = \hat{E} / \hat{H}$, the hazard quotient (HQ). Both the TER and the HQ are used as estimators of risk in pesticide risk assessment.
- *Trigger value.* Although absolute certainty is not possible, we may have high confidence that a substance or process presents a hazard of such low significance that it is deemed to be effectively zero. Similarly, we may have high confidence that exposure to a hazardous substance or process is so unlikely that exposure is

judged to be effectively zero. In these cases, we can judge risk to be acceptable on the basis of 'no hazard' or 'no exposure'. When both hazard and exposure have been estimated to be significantly different from zero, or when there is high uncertainty whether one term approximates to zero, risk needs to be calculated using the estimator of risk. The value of the estimator will be used to make a decision. One way to make decisions is to set a trigger value of the risk estimator, such that estimates greater than the trigger value lead to (trigger) one course of action, whereas those below the trigger value lead to a different course of action. Trigger values are used commonly in tiered testing schemes (see below), in which risk is estimated under progressively more realistic exposure scenarios. In these instances, trigger values are used to determine when testing should stop. For example, in some risk assessment schemes, if the TER exceeds 100 in a laboratory study of the effects of a pesticide on a certain species (indicating that the LC_{50} is at least 100 times greater than the worst possible exposure following normal use of the chemical), no further testing of that pesticide on that species is required (e.g. EPPO, 2003). Clearly, although the use of triggers to decide whether more information is needed is part of the risk assessment, the value of the trigger is largely dependent upon the level of risk deemed acceptable and so is a matter for regulatory decision makers.

7.3 Tiered testing and risk assessment

Tiered tests are an efficient strategy to collect the appropriate amount of pertinent data to assess risk. Tiered testing begins by assessing risk from measurements of hazard and exposure under worst-case conditions; for pesticides, the hazard is measured under conditions where contact with the test substance is unavoidable and the exposure may be set as the application rate of the chemical (or even above this level). A value of the TER under these Tier 1 conditions is defined, by whatever criteria, as acceptable risk. If the TER from Tier 1 tests is above this value, the risk is deemed acceptable and further testing is not required. If the TER falls below this value, however, higher tier tests are invoked involving increasingly realistic exposure conditions. Unacceptable risk is known as the trigger value because different actions are triggered depending on whether the TER estimate is above or below this number.

Tier 1 tests are very simple and consequently are often criticised for being highly unrealistic compared with field conditions. However, these tests are not intended to be realistic; their purpose is solely to aid efficient decision making by exposing organisms to unrealistically high concentrations of a substance and so prevent unnecessary higher tier testing of substances (including GM events) that present very low hazard. For example, if a chemical is deemed safe under worst-case conditions (TER fails to fall below trigger threshold) then sufficient information is available to make a decision and no further information is required for the purposes of risk assessment. If a Tier 1 study indicates an unacceptable risk, then further higher tier tests that introduce more realism can be made. A new trigger value that accounts

for the greater realism of the test is set, and decisions about the acceptability of risk are made in the same way as at lower tiers.

The tiered testing approach has been used widely in the testing of pesticides. A more-or-less standard procedure is now used and accepted within the European Union (e.g. Candolfi *et al.*, 2000; EPPO, 2003). Several authors have called for a tiered approach to risk assessment of GM crops (Poppy, 2000; Wilkinson *et al.*, 2003a), although few have advocated that risk assessment should seek the minimum necessary information to make a decision and stop testing at Tier 1 should acceptable risk be demonstrated. Exceptions are tiered environmental safety tests that are used in the United States to assess the risks to non-target organisms of plants expressing pesticidal proteins (USEPA, 2001). US legislation regards pesticidal proteins as analogous to synthetic chemical pesticides and therefore frameworks for testing chemical pesticides can be easily adapted for assessing the environmental risks of these substances (Dutton *et al.*, 2003).

As more constructs are considered and as construct combinations grow by transgene stacking, the demands on the regulatory infrastructure will also increase. It is therefore realistic to expect that at some point there will be a move towards more efficient risk assessment procedures. Under these circumstances, the routine termination of assessments whenever the TER threshold is exceeded at Tier 1 seems an attractive option.

7.4 General requirements for assessing risks of gene flow from transgenic crops

Risk assessment must begin by defining what we are concerned about; in other words, we have to define the assessment endpoints. Next, it is necessary to analyse existing data to decide whether the proposed course of action, the cultivation of a transgenic crop, poses a conceivable risk to the assessment endpoints. If the data indicate high likelihood that the assessment endpoint will not be exposed to plants containing the transgene, or that the transgene poses no hazard, then we decide that there is no detectable risk and no further data are required. Conversely, if existing data indicate with high certainty that the hazard and exposure are unacceptable, then again no further data may be needed to make a decision (presumably rejection of the application or request for risk management). Thus, it is only when there are no or insufficient data on the hazard and exposure to define risk sufficiently clearly for a decision to be made that new experimental studies are required. In these instances, the initial studies should be carried out under worst-case conditions (Tier 1) to maximise our confidence that our conclusions apply to all conceivable situations in the field.

The identification of assessment endpoints, possible hazards and routes of exposure, and, if necessary, suitable Tier 1 tests, is the 'problem characterisation' phase of the risk assessment; clarity and precision at this stage is vital to focus the collection of data and gain agreement for decisions based on the risk assessment. Experimental studies performed to support the risk assessment aim to estimate the

expected exposure and hazards, combine these into an estimate of risk and so enable decisions based on whether the estimate of risk predicts an acceptable or unacceptable change in the assessment endpoint. Tests should start with unrealistically high exposures and increase realism until acceptable risk is demonstrated. Once acceptable risk is shown, testing should stop; if unacceptable risk is demonstrated, in some instances risk management can be used to reduce the risk to acceptable levels. If the risk cannot be managed, the risks are unacceptable and the crop will not gain registration (see Raybould, in press(a), for a more detailed discussion).

7.5 Assessment endpoints

It is possible to imagine an enormous number of phenomena that could change after the cultivation of a transgenic crop. If one adopts the position that ‘all ecological change is bad’ and applies the precautionary approach to an extreme, then it is possible to argue that it will never be possible to be certain about the environmental safety of almost any GM cultivar. Moreover, one will always be open to the criticism that, with the number of possible environmental changes for each construct being limited only by the imagination of the assessor, by definition the risk assessment has not considered unforeseen hazards (so-called ‘unknown unknowns’).

However, science is concerned with what is *probable*, rather than what is *possible* (Peters, 1991). Unless one adopts this position, risk assessment becomes entirely unfocused and the regulatory system is paralysed, which is, no doubt, the intention of at least some who advocate precaution because of unknown unknowns. On the other hand, it is vital that the risk assessment considers ecological impacts of high significance. Thus, risk assessment needs to concentrate on probable events that we care about, rather than those we do not. Therefore, a system is needed to identify suitable representative assessment endpoints to avoid dissipating effort studying events that are unlikely to occur and about which we care little.

How are we to derive assessment endpoints for the potential harmful effects of gene flow from transgenic crops? A consensus is that ‘the transgenic process presents no new categories of risk compared to conventional methods of crop improvement’ (National Research Council, 2002). In the future, there may be some exceptions to this rule (e.g. entirely synthetic transgenes with novel function), although the principle will always apply to the vast majority of cases. Therefore, the assessment endpoints for risk assessment of gene flow from transgenic crops can be the same as for conventional crops and other potentially invasive plants.

Pimentel *et al.* (2001) reviewed the potential environmental threats of alien plants. They concluded that the main problems caused by invasive plants, other than weeds of crops, are

- Displacement of native plant species (and presumably other taxa that use those plants as food, shelter, etc.)

- Physical changes, including reduced water supply, increased frequency of bush fires and changed nutrient cycles
- Detrimental effects on recreation (this is a particular problem with aquatic plants that affect fishing, boating, swimming, etc.)
- Loss of yield in semi-natural pastures
- Costs of control

The problems identified by Pimentel *et al.* are probably not an exhaustive list of possible harmful effects of transgenic wild relatives, although they are likely to be the most important. Also, they are very close to being operational assessment endpoints; for example, physical changes could be quantified in terms of litres of water, or area burnt; displacement of native species could be defined in terms of population sizes of taxa with legal protection, and so on.

Predictions of changes in assessment endpoints are probably the limit of science in environmental risk assessment (see Conclusions). Decisions based on these predictions will reflect the relative importance society places on these endpoints and on the potential benefits of the transgenic crops.

7.6 Hazard assessments

There are three broad ways in which a transgenic plant could pose harm to the assessment endpoints listed above. First, the product of the transgene could be toxic, which could lead to the displacement of native species. Second, all the assessment endpoints discussed above could be detrimentally affected by expanded ecological range of crop wild relatives following introgression of a transgene; in other words, the hazard is invasiveness. Third, stable recruitment of the transgene into the wild relative may detrimentally change its population dynamics, such that a cohabiting species declines or becomes locally extinct.

7.6.1 Toxicity of transgene product

Predicting toxicity of a transgenic protein is relatively straightforward. Standard Tier 1 laboratory methods are available to expose representative non-target organisms to high doses of protein (e.g. Hellmich *et al.*, 2001; US EPA, 2001; Romeis *et al.*, 2004) and often the range of toxicity of a protein can be predicted from its mode of action (e.g. Schnepf *et al.*, 1998; Lee *et al.*, 2003).

7.6.2 Enhanced invasiveness of transgene recipient

Predicting whether a plant will become invasive is rather more difficult to evaluate than whether it will become toxic. While it is possible to predict with reasonable

confidence that 1 out of every 1000 plant species introduced into a country will become invasive (Williamson & Fitter, 1996), it is not possible to predict which species they will be. Many authors have tried to predict whether plants will be invasive based on phenotypes (e.g. Baker, 1974) or genotypes (e.g. Gray, 1986), but the success of these predictions thus far has been low (Williamson, 1994). This has led some authors to despair about whether we can predict the invasiveness of transgenic crops or wild relatives because data and mathematical models will always be insufficient to cope with uncertainty (Kareiva *et al.*, 1996).

The conclusion that there is nothing we can do to assess the invasiveness of GM crops and their relatives seems unduly pessimistic. First, we should remember that risk assessment is not scientific research; our purpose is to collect data that enable us to make a robust decision, not predict with great precision the population size of an introduced plant several years into the future. Second, we are not starting from scratch, either in terms of collecting data or in assessing the risks we are prepared to accept. We know a great deal about the growth of non-transgenic crops and, because they are grown widely with little regulation, it seems that we are prepared to accept the risks they pose. A corollary of these assertions is that if the hazards posed by a transgenic crop are no greater than those posed by existing conventional varieties of the same crop, then the risks should be acceptable (assuming that the transgenic crop is grown in the same places and under the same conditions as the conventional crops, i.e. exposure is unchanged).

How are we to determine whether the hazards of a transgenic variety are no greater than those of the varieties it is intended to replace? With regard to predicting the likelihood of invasion, it is important first to identify the traits that currently restrict the ecological range of the target species. The reasoning being that if a characteristic conferred by a particular transgene can release the species from a key constraint, it will be free to occupy new habitats or new niches. There are three broad classes of recipient plants that could be affected in this way: feral or volunteer populations of the crop itself, weedy relatives of the crop and wild relatives of the crop occupying natural or semi-natural habitats. Clearly, the factors that restrain each of these plants to their respective environments will differ and so they should each be considered separately.

So how is it possible to determine whether the acquisition of a particular transgene will enable a recipient to broaden its ecological range? The most direct approach is to focus on the characteristics conferred by the transgene. However, it should be remembered that there are phenotypic and physiological features that are associated with the purpose of the transformation event (intended effects) and others that are by-products of transformation (unintended effects); the latter may result from pleiotropy, epistasis, somaclonal variation and so on. Evidently, intended effects of the transgene are far easier to assess with confidence because they can be simulated in experiments, or existing data can be related to the intended phenotype.

A simple but robust approach is to mimic this effect experimentally. For example, Raybould *et al.* (1999) assessed whether transgenic insect- and mollusc-resistant varieties of oilseed rape (*Brassica napus*) were likely to be more invasive than

conventional varieties; the effects of the putative transgenes were simulated by spraying cultivated plots of rape with insecticide and/or molluscicide. Once the rape had reached maturity, cultivation was stopped, but pesticide treatments were continued. Although insect and mollusc damage was significantly higher in the untreated control plots, the number of oilseed rape plants subsequently recruited into the sprayed plots was no different from that in the unsprayed plots. This result suggests that pressure from insects or molluscs is not limiting population growth in oilseed rape under conditions that simulate a ruderal environment, and so the introduction of transgenes conferring resistance to these herbivores is unlikely to result in expansion in population size or range.

This type of experimental strategy could equally be applied to natural or semi-natural settings when the transgene has a similarly direct effect but may not be as powerful in circumstances where the constraining factor is likely to be stochastic (e.g. disease) or difficult to manipulate in the natural setting (abiotic pressures such as frost tolerance). On the other hand, sometimes no experiments may be required: predicting that the intended effects of transgenes for herbicide resistance will not increase the invasiveness of crops in areas where no herbicides are applied is one such case. Thus, there is clear variability between groups of transgenes that share common features (e.g. those conferring virus-resistance or others generating enhanced oil quality) in their scope to expand the ecological niche of a species and also in our ability to test this property by direct experimentation. In those cases where experimental manipulation is impractical under conditions that simulate the native habitat, it could be possible to adopt a correlative approach. Here, the aim would be to determine whether the limits of the distribution of the species are associated with a change in the feature directly affected by the transgene. For instance, if the limits of the population are invariably or sometimes set by a change in soil salt concentration, or with mean soil moisture, then it could be reasoned that transgenes conferring salt or drought tolerance could lead to expansion of its ecological range. Conversely, the absence of any gradient across the population boundary or associated with sites of high and low plant density suggests that these transgenes are unlikely to result in range expansion.

Unintended hazards of transformation can also be tested experimentally using the argument that the GM cultivar need pose no greater risk than its non-GM counterpart. One method is to measure invasiveness directly by sowing seeds of transgenic and non-transgenic crops into a variety of habitats and comparing the growth rate of the result populations of crop plants. This method was adopted by Crawley *et al.* (1993, 2001) who showed that certain herbicide-tolerant varieties of potato, sugar beet and oilseed rape were no more invasive than their conventional counterparts.

The 'Crawley style' experiments have been criticised (e.g. Kareiva *et al.*, 1996) because they did not cover a sufficient number of habitats or years to reduce uncertainty sufficiently. This is an easy criticism to make, especially when it is not made clear what a sufficient data set would comprise. Of course, it is true that further experiments will reduce uncertainty; however, further experiments will also introduce

delay. William Rodgers, John Graham and Jonathan Wiener (quoted in Cross, 1996) give an interesting perspective:

- The ‘insatiable pursuit of data facilitates delay; any decision dependent upon extensive data gathering promises to be long in incubation and short on results’ (Rodgers).
- One must balance ‘the value of more information to better decisions and the cost, including delay of decisions’ (Graham and Wiener).

In other words, collection of more data will delay a decision and may not improve the decision because the problem becomes more complex. Even if a better decision is made (i.e. one has more confidence in the prediction), the overall environmental risk may not have been reduced if the delay prevented the introduction of a technology that poses a lower risk than current practice. Therefore, calls for extra data should not be made lightly and collection of extensive data sets may not be the best route to reduction of environmental risk. To take the lessons from the assessment of the hazards of pesticides under worst-case exposure, the comparison of invasiveness should take place in conditions most likely to lead to spread of the plants. Crawley *et al.* compared population growth rates in plots that were cultivated to maximise the chances that the crops would survive in the natural habitats and still the crops did not persist, let alone invade. Surely, this is sufficient to decide that the invasiveness (hazard) of these crops is low and allow scientists to do experiments that are more productive than sowing crops into other natural habitats and observing them for another 10 years.

A more pragmatic criticism of the experiments of Crawley *et al.* is that they are expensive and time-consuming. It might be possible to use data collected during the development of the crop to reach the same conclusions. During development of transgenic crops, their performance is compared with conventional varieties in a series of multi-location agronomic field trials. USDA-APHIS (White, 2002) suggests that such trials can be used to assess whether the transgenic variety is more likely to be invasive (or become a volunteer weed). Suggestions of traits to measure include the following:

- Reproduction and survival characters
 - For example, lifespan, vegetative biomass, over-wintering capacity, flowering behaviour, seed production, seed dormancy, germination, seedling survival, outcrossing frequency, pollen viability, dispersal ability (panicle shattering, etc.)
- Adaptation to stress
 - Biotic – pathogens, herbivores, other plants
 - Abiotic – atmospheric pollutants, nutrient deficiency, temperature extremes, drought, flood
 - Pesticides
- Nutritional composition (undefined)
- Levels of natural toxicants (undefined)

APHIS concludes that ‘observed changes may warrant further in-depth studies’. The rationale presumably is that if no differences in these traits can be observed, the transgenic crop is unlikely to be more invasive than the conventional crop. Notice that the requirement is not to predict *how* invasive the transgenic crop is, rather whether it is *more* invasive than the conventional crop that, by implication, poses acceptable risk. In other words, we are developing a tool for risk assessment, not carrying out research in theoretical population biology.

Nevertheless, this is a pragmatic argument that relies on a ‘scatter-gun’ assessment of features that *could* define ecological limits of the crop and so lead to increased invasive properties. In practice, therefore, the list of traits assessed in this way should be continually refined and weighted in the light of information suggesting those traits that are or are not important in limiting the ecological range of the assessment endpoint species.

7.6.3 *Transgene-induced changes to community of the recipient*

The third group of ecological hazards relates to unwanted perturbations within the existing habitat of the wild relative or feral crop. That is, scenarios where the presence of the transgene influences plant abundance within in its own habitat, and without enabling invasion of new habitats, nevertheless causes unwanted ecological change. There are few plausible scenarios where acquisition of a transgene impacts negatively on the population size of the recipient. On the other hand, there are many interactions with cohabitant species that could lead to plausible ecological hazards. For example, a transgene could enhance the ability of a recipient wild relative to compete more effectively with another species occupying the same habitat, thereby causing decline of the latter. Alternatively, the presence of transgene-mediated resistance could lead to displacement of an herbivore onto another plant species, possibly causing decline in either the alternate food source or of the herbivore itself. In short, a change in abundance within its own habitat is likely to cause changes in community structure.

If one adopts ‘an all change is bad’ doctrine, then any transgene that could alter a recipient wild species’ interaction with other species should be viewed with caution. The corollary of this view is that not only should any transgene conferring a hypothetical selective advantage be viewed cautiously, but also those that only impact on other species, either directly (e.g. passage of toxic product producing insect resistance through the food chain) or indirectly (e.g. by changing availability of an herbivore as a food source for a specialist predator).

Given the complexity of ecological interactions even within simple communities, it seems a daunting prospect to attempt to predict how one particular trait could influence all of the species occupying the habitat. The nub of the problem lies in the multitude of possible assessment endpoints that need to be anticipated if the sole criterion for inclusion is a significant difference from the non-GM counterpart. This issue will become increasingly germane as the number of constructs increases

and as trend for stacking transgenes continues. Once more, however, it should be remembered that the goal is risk assessment and not the more demanding discipline of predictive ecological science. In reality, not all ecological perturbations are equally important and anticipated changes need to be placed in context with those arising through other causes (farm practice, use of autumn-sown cultivars, urban development, etc.). For example, a secondary effect causing minor local decline of a particular herbivore widely viewed as a pernicious pest is not comparable with local extinction of a globally rare species. Thus, we need to focus our efforts on identifying potential hazards relating to organisms and communities that we care about. For this, we need a system or a guiding principle, such as 'are we sufficiently concerned about the fate of this organism to consider other routes by which it could be affected that do not involve GM crops?' If the answer is 'no', then effort should clearly be switched to other, higher priority organisms. The natural progression of this line of thinking is that if it is specific aspects of the environment that we are concerned about, then perhaps there is a need to start placing GM risk assessment into a broader context in which other routes by which such changes occur are also considered. Otherwise, there is a real danger that while we are concentrating on evaluating whether GM causes realisation of a particular hazard, we may overlook the possibility that damage may be more likely to occur by another (probably unregulated) route. This is a matter for future debate on how we manage our landscape.

From a practical viewpoint, we need a system to identify and rank the most significant assessment endpoints for risk assessment. The simplest and most direct way of achieving this goal is to centre attention on the species and communities that have value on the basis of current legislative protection. Other lower level ranking could be invoked on the basis of national or regional scarcity, phylogenetic isolation, cultural importance and ecological importance (e.g. keystone species). Once a list of important species/communities has been assembled, effort should then focus on performing case-by-case risk assessments to evaluate how particular transgenes could impact upon these targets.

The first task is clearly to identify those species and communities that are exposed to the feral/volunteer crop or else to the weedy or wild relative into which a transgene can move by gene flow. Rather surprisingly, there is a remarkable paucity of information on plant and animal species that co-occur with crop wild relatives or with feral populations of the crops themselves. One reason for this is that many relatives occur only as weeds over much of their range and so are generally associated with other common weeds and farmland pests. A good example of this is *Aegilops cylindrica* (jointed goatgrass), which grows as a weed of wheat and as an adventitious ruderal of waste ground in the United States and readily forms hybrids with bread wheat (Morrison *et al.*, 2002). In these instances, one can generally refer to weed floras and farmland surveys to identify the more common associated organisms, although targeted surveys may be required to identify the more endangered associated taxa.

Community associations become more complex outside the agricultural environment and it is when relatives or crop escapes occupy natural or semi-natural habitats that information can become difficult to obtain. Clearly, direct studies of the species associations of wild relatives of crops would provide the most germane source of information, although these are surprisingly rare. Mitchell and Richards (1979) provide one useful exception, in which they list coincident plants associated with wild populations of a close relative of oilseed rape in the United Kingdom, *Brassica oleracea*. More general species associations used to describe vegetation types are more widely available (e.g. Rodwell), but typically lack the less frequent and endangered species that would constitute targets for any hazard assessment. To some large extent then, further work is still required to allow systematic identification of associated non-target species that are most vulnerable to hazards arising from gene flow. Accumulation of this (conceptually) rather simple set of data must be viewed as a priority.

Once vulnerable associates are identified, of assessment endpoint identification and prioritisation is very much simplified, with emphasis being placed on hazards that detrimentally affect population size and distribution of species deemed to be important in some way. As mentioned above, this should be based on national priorities and the highest priority species perhaps most readily assigned by those that already afford legislative protection (for example, Council Directive 92/43/EEC on the conservation of natural habitats and of wild fauna and flora in the European Union). In many cases, probably most, there will be no associated plant or animal species that merits legislative protection or ecological concern because feral crops and relatives of crops are often agricultural weeds/volunteers or else inhabit disturbed habitats. Risk assessment should therefore focus on the exceptions.

The subsequent risk assessments attempt to evaluate circumstances leading to the decline (or spread in the case of a pest or pernicious weed) of the target associate. In cases where the associate is another plant species, the key element of this task is to determine whether acquisition of the transgene will change the ability of the transgenic crop wild relative to compete with the associate.

This could be evaluated directly through pairwise competition trials, although in some instances such experiments may lack the subtleties of competition in the wild or may be impractical because of difficulties in obtaining sufficient material of the endangered associate. One option to address this problem is to use field comparisons of the life history of the recipient wild relative or of the feral crop with the GM crop. Here, the aim would be to determine whether there are significant differences between the GM and non-GM plants for a measurable parameter such as population growth rate. This could be achieved using matrix models (e.g. Bullock, 1999). In effect, modelling refines the hazard because not all increases in growth or reproductive output lead to increases in population growth rate. For instance, if a transgenic wild relative or crop produces more seed than its conventional counterpart, this does not necessarily make it more invasive; it may be that seed production does not limit the population growth rate of the conventional crop (Bergelson, 1994). The employment of a matrix modelling approach will help diagnose such problems

since elasticity analyses (e.g. Davis *et al.*, 2004) can be used to determine which stages in the life history are most and least influential in population growth. While this strategy is attractive for GM crop to non-GM crop comparisons, practical difficulties may arise in the comparison of GM and non-GM wild relatives. This is partly because of the need to generate GM plants of the relative, but largely because of the wide capacity for different genotype-environment combinations, meaning that selecting representative genotypes and habitats could be difficult. It therefore seems that comparative life history experiments should be performed on GM and non-GM crops, and may only sometimes be practical for wild relatives. Alternatively, it may be possible to mimic the effect of the transgene in natural populations or in simulated natural populations and observe the influence on population growth rate and other life history parameters. In all cases, it should be remembered that the aim is simply to determine whether there is a detectable difference, not to quantify these differences.

Hazards arising from interactions between a feral GM crop or GM wild relative and an herbivore or higher trophic level animal associate can be relatively readily evaluated using a tiered experimental approach, provided *ex situ* culture of the associate is possible. Alternatively, it may be possible to use a more common relative of the endangered species as a surrogate.

In summary, the hazards of GM crops and of introgressed GM wild relatives can be assessed. Intended effects of the transgene can be predicted from laboratory toxicity studies, efficacy studies of the crop and other data such as mode of action of the protein. Hazards of these effects are relatively easy to combine with exposure assessments to assess risk. Unintended phenotypic effects can also be assessed from agronomic data and, if necessary, their effects modelled. The question is whether these crop data are sufficient to assess the hazards posed by the transgene introgressed into the genetic background of a wild relative.

Two important points should be remembered. First, during development of a transgenic variety the transgene is moved among many crop genetic backgrounds and its genetic and phenotypic stability is assessed. Events that show instability or unacceptable unintended effects are discarded. Second, we are not assessing the absolute effect of the transgenic crops; rather we are comparing them with the effects of conventional crops. Therefore, if the transgene is stable and has no unintended effects in the crop, and there are no instances of problems with hybrids of the conventional crop, then it could be argued that unintended effects of the transgenic crop pose no additional hazards compared to the conventional crop. However, a stronger argument for no greater risk may be made from assessments of exposure.

7.7 Exposure assessments

Exposure to hybrids between transgenic crops and wild relatives can be considered in two stages. Stage 1 is the likelihood that the transgenic crop can form hybrids with wild plants in the territory covered by the risk assessment. If hybrids can be formed,

then the exposure assessment should consider where the hybrids will occur and whether these sites are in areas that would potentially cause harm to the assessment endpoints.

The possibility of hybrid formation (i.e. the sexual compatibility of a crop and a wild relative) can be assessed using a tiered approach as advocated by Raybould (in press(b)).

Tier 1: Test for hybrid production using laboratory methods (hand pollination, embryo rescue, etc.)

No hybrids, stop testing; hybrids, go to Tier 2

Tier 2: Test for spontaneous hybrid production (lab/field)

No hybrids, stop testing; hybrids, go to Tier 3

Tier 3: Search for naturally-produced hybrids

No hybrids, stop testing; hybrids, carry out quantitative risk assessment

If hybrids are found then data on the distribution of the wild relative can be used to refine the exposure assessment. It is important to remember that the risk assessment seeks to predict the effects on the assessment endpoints, not necessarily an accurate prediction of actual exposure. In other words, we do not need to predict transgene frequency, only whether wild relatives occur in areas where they could affect the assessment endpoints. Therefore, if the wild relative does not occur in areas of value for nature conservation, recreation or water supply (see list of endpoints of Pimentel previously listed), then we would consider that to be no exposure, regardless of the fact that some hybrids may form in other areas provided there is negligible scope for secondary transgene spread to these areas.

7.8 Proposed scheme for assessing the environmental risks of gene flow

We have shown that it is possible to use tiered methods to assess the hazards of, and the exposure of, assessment endpoints to transgenes in wild crop relatives. If we take current agricultural practice as posing acceptable risk, then a risk assessment can be based on the toxicity of transgenic proteins and comparisons of transgenic and conventional crops in a tiered approach to identify hazards and quantify risks associated with crop relatives.

The simplest way to demonstrate acceptable risk is to show with high certainty that the transgene poses no hazard to the assessment endpoints or that the assessment endpoints will not be exposed to the transgene. Lack of detectable significant hazard can be shown by the following:

- No predicted toxic effects of the protein to native non-target species, particularly those of natural conservation interest
- The intended effects of the transgene do not increase the likelihood of wild relatives becoming more invasive

- The transgene is stable in a range of crop genotypes and has no unintended phenotypic effects
- The absence of cross-compatible wild relatives or feral crop populations
- The absence of legally protected or environmentally important plant or animal species associated with the wild relative or feral crop populations

Lack of exposure can be demonstrated by the absence of hybrids in areas where harm to the assessment endpoints could occur.

If hazard and exposure cannot be ruled out with high certainty, the scheme can be made quantitative by assessing the likelihood of hybrid formation and introgression, and prediction of the changes in the assessment endpoints under different amounts of exposure (transgene frequencies). Development of such a scheme is not easy, but the essential elements can be identified as follows:

1. *A prediction of equilibrium transgene frequency in areas that affect the assessment endpoints.* The beginning of such a prediction was made by Wilkinson *et al.* (2003b) who used remote sensing, extensive field surveys and statistical models to derive a map of the probability of hybrid formation between *Brassica napus* and wild and weedy *Brassica rapa* in the United Kingdom. However, this is only an estimate of the first step in the introgression of the gene.
2. *A prediction of the changes in population dynamics of the wild relative under different transgene frequencies.* This is a very difficult problem as the population dynamics will affect the transgene frequency and vice versa. Conceptual models are available to describe these interactions, but parameterisation of the models is extremely difficult and at a rudimentary stage even for intensively studied species and traits (e.g. Raybould & Moyes, 2001).
3. *A prediction of the changes in the assessment endpoints because of the changed population dynamics of the wild relative.* Again, this is an extremely difficult problem. First, large, long-term sets of data are required to determine the baseline condition of the assessment endpoint. For example, if the endpoint is the population size of a plant, we would need to know not only the current population size but also the variability and long-term trends in the population size; 30–50 time points may be required to achieve robust predictions of long-term trends (Poole, 1978).
4. *An estimator of risk.* How should hazard and exposure be combined into a single estimate of risk? This is not an easy question to answer, even in concept, as hazard and exposure are not independent (the interaction between population dynamics and gene frequency described above).
5. *An unacceptable level of risk.* Strictly, this is outside the scope of the risk assessment and should be set by decision makers. However, the amount of certainty about the predictions, and hence the safety margin to keep an acceptable amount of risk, will depend on the elements listed above.

A rigorous quantitative assessment of risk requires an enormous amount of data and development of theory. It is questionable whether such effort is worthwhile except in rare cases: the cost of the studies and the delay in introducing a crop may be prohibitive, and regulators may feel that there is too much uncertainty to make decisions based on such a scheme. Therefore, we envisage that risk assessment of gene flow from transgenic crops to wild relatives will be based on no demonstrable hazard and/or exposure for the foreseeable future.

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8 Quantifying exposure

Jamie P. Sutherland and Guy M. Poppy

8.1 Introduction

Gene flow from GM crops to wild and weedy recipients is a likely event, but whether gene flow matters ecologically depends entirely upon its consequences. Gene flow is always the result of the combination of genetic material from individuals with different genetic backgrounds, e.g. between crop cultivars or populations of wild recipient plants. The critical questions are, surely, whether these genetic combinations can persist in the environment in subsequent generations, and, if they do, what if anything is their ecological significance. This chapter will provide a synopsis of the variety of techniques and approaches to quantifying exposure as a component of risk assessment for gene flow from transgenic crops to wild or weedy species.

The development and introduction of novel transgenic traits and varieties (Dunwell, 2002) will, in the near future, pose an enormous challenge for scientists, should separate gene flow assessments be required for each variety and trait. Therefore, a more logical approach to GM risk assessment, which is as generic as possible, is to study the consequences of gene flow. Hazards need to be defined precisely and ranked, with emphasis on quantifying the elements of exposure. It will be important to distinguish between the detrimental environmental effects associated with introgressed transgenes and those caused by other anthropogenic effects in the agroecosystem.

This chapter will be concerned predominantly with insect-resistant transgenic crops. Most emphasis will be placed on how we can measure exposure to insect-resistant transgenic plants; many of the examples will be drawn from research on conventional crop cultivars rather than plants containing introgressed transgenes, because of the paucity of research on the consequences of transgene expression in wild relatives.

Of course, an important benefit of insect-resistant transgenic crops over conventional insecticides is the high specificity of the insecticidal proteins conferred. Moreover, effects on non-target organisms should in theory be minimal (MacIntosh *et al.*, 1990) should gene flow occur. At the time of writing, while several types of toxins (including proteinase inhibitors, lectins, chitinases and cholesterol oxidase) have been experimentally introduced into crop plants, only *Bt* has gained commercial approval. *Bt* uses a group of bacterial proteins known as the δ -endotoxins derived from the bacterium *Bacillus thuringiensis*. Over 100 types of δ -endotoxin have been discovered, each of which is relatively specific to Lepidoptera or Coleoptera (Peferoen, 1997). The crystals of pure protein endotoxin contained by *Bt* have been used for many years in agriculture as a microbial spray, mostly on organically grown

crops on which synthetic insecticides cannot be used. However, biotechnology has made it possible to produce a single *Bt* toxin inside plant cells, increasing the physical targeting and hence the efficacy of the treatment, eliminating or reducing the need for insecticide spraying.

To date, transgenic crops have been created possessing resistance to European corn borer, *Ostrinia nubilalis*, corn earworm, *Heliocoverpa zea*, southwestern corn borer, *Diatraea grandiosella*, and recently the corn rootworm complex *Diabrotica* spp. in maize; Colorado potato beetle, *Leptinotarsa decemlineata*, in potato; tobacco budworm, *Heliothis virescens* in tobacco, and cotton bollworm, *Helicoverpa armigera*, in cotton. In theory, should a wild relative also confer resistance to these insects then this specificity should mean that the effects of exposure of non-target organisms to the proteins would be negligible in contrast to broad-spectrum insecticides (Schuler *et al.*, 1998).

The ultimate purpose of this chapter is to provide the reader with a roadmap for assessing and quantifying exposure in plants where gene flow might have occurred and to propose a more generic strategy for risk assessment that can be used for a wide range of crops in a range of agro-environments. Methodologies are currently being developed to assess the potential impact of transgenes on plant fitness to provide less expensive alternatives to the Farm Scale Evaluations (FSEs) and PROSAMO-type experiments (see Section 8.7). These include targeted experiments, tiered experiments, simulated transgene expression and modelling (Linder & Schmitt, 1994; Hails, 2000; Wilkinson *et al.*, 2003), which will all be described in the subsequent sections of this chapter.

8.2 Defining risk, hazard and exposure

It is important to clarify what is meant by 'risk', 'hazard' and 'exposure' before discussing how we can quantify exposure to GM plants. Too often, the words 'risk' and 'hazard' are used interchangeably and confusion often results. Such confusion between hazard and risk led to the well-publicised article suggesting that pollen from transgenic maize could bring about the extinction of the monarch butterfly, even though the experiments were simplistic 'worst-case' laboratory studies conducted in Petri dishes (Losey *et al.*, 1999).

The most useful available working definitions of these three terms are provided by the UK Department of the Environment (now Defra). A hazard is defined as *a situation that in particular circumstances could lead to harm*, while risk is *a combination of the probability of occurrence of a defined hazard and the magnitude of the consequences of the occurrence* (DoE, 1995). Identification of a hazard is an essential component of risk assessment, but in order to quantify the risk, the likelihood of the hazard being realised or exposure needs to be accurately determined (Figure 8.1). Exposure represents *the probability that the hazard will occur and so is readily quantifiable, provided the hazard is clearly defined* (DoE, 1995).

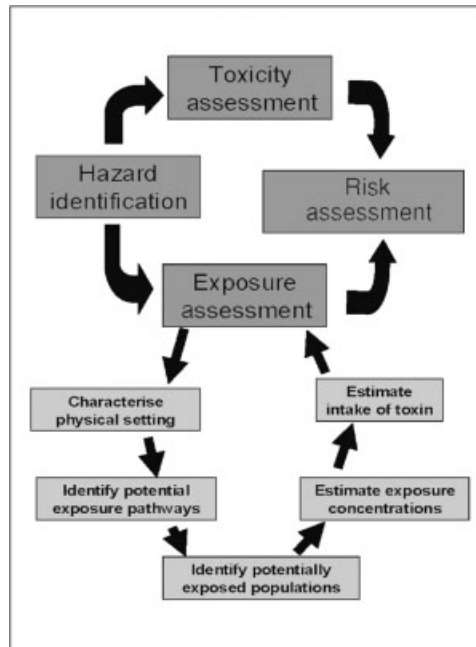


Figure 8.1 Steps in quantifying exposure and how it relates to hazard identification and overall risk assessment.

An ecological risk assessment (ERA) uses these three terms and requires estimates of the probability of harm to an organism. ERAs generally consist of three phases: (i) the problem formulation (i.e. hazard identification), (ii) analysis (including receptor identification, exposure assessment and toxicity assessment) and (iii) risk characterisation. An ERA requires the specification of a relationship between the stressors (physical, chemical or biological entities that induce a negative or detrimental response) and the receptors (ecological entities exposed to the stressors) (US EPA, 1998). In an optimal ERA, risk estimates should be derived and measured against specific ecological endpoints (Poppy, 2003). The use of assessment and measurement endpoints allows clear integration between the collection of data and the management goal, and thus allows risks to be characterised, assessed and managed. A similar approach for the estimation of gene flow into wild recipients from GM plants would be a significant step forward on the current risk assessment framework, and could allow scientists involved in detailed laboratory studies to fit their research into the bigger picture (Poppy, 2003).

A large proportion of early GM risk assessment consisted of investigations that were merely hazard identification studies (Poppy, 2000) and include the well-publicised work of Losey *et al.* (1999) (see Section 8.6.1). After much public and media outrage, this was followed up by exposure studies (Oberhauser *et al.*, 2001; Sears *et al.*, 2001; Stanley-Horn *et al.*, 2001), which quantified the probability that

the specific hazard would occur and as such they permit a thorough assessment of risk (Gatehouse *et al.*, 2002). It is unfortunate that particular short-term or laboratory studies identifying possible hazards, such as the monarch butterfly example, have, in the past, received more media attention than thorough ecological field-based studies that either evaluate exposure to a hazard or assess fitness over several generations (Gray, 2004).

In most instances, the relationship between crop and hazard is highly complex and so a risk assessment of gene flow and its consequences would be far more protracted. Furthermore, as the complexity of GM cultivars increases, the number of associated hazards might become too numerous for all to be identified and assessed at a rate that keeps pace with submissions. An unstructured approach to hazard identification under these circumstances might concentrate on only those traits with immediate negative effects rather than those that might have more serious long-term ecological consequences. We therefore propose that a structured approach be adopted for hazard prioritisation so that the risk assessment is as generic as possible. To some extent, this is a formalisation of the approach already adopted by some regulatory bodies but, in addition, we advocate that data be assimilated in a coordinated fashion to provide information on the relevant geographic scale for legislation.

It should be borne in mind that the public may perceive risks very differently. Thus because the hazards linked with GM are often so outrageous, the perceived risks are too great for acceptance of the technology. These ideas, discussed by Professor Peter Sandman, are possibly the reasons why newspapers, pressure groups and politicians tend to react to the outrage rather than to the probability phenomenon (Sandman, 1988). However, in this chapter we will adhere to the scientific assessment of exposure and how it can be used to assess risk of gene flow from GM crops. Where appropriate, the broader issues will be alluded to but the more complex debates about risk and the precautionary principles are beyond the remit of this chapter (but see Myhr & Traavik, 2003, for a review).

8.3 Exposure trees

In developing risk assessments and determining the consequences of gene flow, there are several options available. The first technique is to use an 'exposure tree' that focuses on the exposure term. In developing an exposure tree, it is necessary to assess completely the possible routes of exposure and deduce which taxa might be exposed to the transgene protein, should introgression occur. Potential routes of exposure may include contact of non-target organisms with the leaves, floral parts, stem, pollen, seeds and fruits of the introgressed recipient, or in the rhizosphere surrounding the roots. It must also be remembered that exposure to the transgene protein may be via direct contact by consuming plant material or via indirect contact by consuming other organisms that have ingested the toxins.

Exposure can be divided into a series of steps in a sequential pathway and the entire pathway must be completed for the hazard to be realised (Wilkinson *et al.*, 2003).

In an exposure tree, exposure is therefore quantified as the cumulative probability of completing the pathway. If the cumulative probability reaches zero at any point in the pathway, then the risk of hazard realisation is negligible. As agroecosystems are complex ecological systems, the exposure pathway is not always as simple as a linear pathway. For any transgenic plant, there is typically a complex tree of interconnected pathways that could, in theory, lead to a different hazard. The final stages of an exposure pathway, i.e. the effects on tritrophic interactions, assume the occurrence of transgene introgression and spread in the environment.

There are two types of exposure tree in existence. Event-tree analyses work through a sequential series of steps in a logical causal chain (Haimes, 1998). Event-tree analysis is not necessarily conditioned on the existence of a known hazard. Starting with an initial event, the next step in the pathway of events can be assessed until the risk probability associated with a hazard can be calculated from the probabilities associated with the chain of events. Event-tree analysis may become particularly useful when risks associated with transgenic plants with many traits are characterised. Fault-tree analyses logically evaluate risk by tracing backwards through a suspected causal chain the many different ways that a particular risk could happen (Haimes, 1998). To do this, it is essential that a specific hazard is known before the analysis can be conducted. This has advantages because the analysis focuses on how the risk occurs and does not waste time and resources evaluating risks that could not possibly occur. By concentrating on the known hazards, the analysis provides a robust strategy for assessing risk. However, it is disadvantageous because it does not allow for the occurrence of unknown or unexpected hazards. Fault-tree analysis is widely used in industry to assess the risk involved in potential failures of technology such as in aeroplane safety (Lloyd & Tye, 1982).

When we are considering exposure to a single known transgenic protein, the most suitable approach is to use fault-tree analysis and employ a tiered approach (see Section 8.4). Thus, the strategy is opposite to that used for evaluating risks of gene flow itself (see Chapter 7), as we are primarily concerned with measuring exposure to a known hazard. This procedure means that research, and so considerable expense, is restricted to hazards with a significant likelihood of occurrence.

This research can either be via controlled a priori releases of a plant expressing the transgene protein and/or experimentation in the laboratory, glasshouse and semi-field. Obviously, a priori releases would deliver the most realistic data for quantifying exposure, but controlled experimentation is the only practical option in many cases (see also Chapter 6). Exposure quantification will almost inevitably require the eventual use of transgenic plants, ideally involving empirical data collection, which could then be input into powerful models for longer term predictions (Section 8.8.2).

8.4 An introduction to tiered risk assessment

Tiered risk assessment, similar to that used in insecticide toxicity testing, typically begins with simple qualitative or comparative methods in the laboratory, almost

always employing worst-case scenarios on individuals whereby the test organisms are given no choice as to whether they feed on the transgenic plant material. This is followed by more quantitative experimentation investigating population or community effects of exposure and concludes with an investigation at the ecosystem or field level. Providing these studies are part of a tiered assessment, such hazard identification does have enormous value because of the importance of initially detecting any direct effects of the protein on the non-target organism.

Tiered risk assessment is strongly advocated to determine the possible effects of an introgressed transgene on non-target organisms such as herbivores, pollinators, parasitoids and predatory insects (Poppy, 2000). This is schematically represented in Figure 8.2. The aims of the tiered risk assessment are first to define the likely exposure patterns, second to identify the major sources of exposure that are driving the associated risk and finally to assist in the development of mitigation strategies to improve the overall risk profile. To support these aims it is necessary to determine the exposure estimates by each major pathway. It is important to bear in mind that a tiered risk assessment differs from the event-tree exposure type approach in that it is more concerned with exposure of non-target organisms to a specific known hazard. This makes the assumption that non-target organisms will encounter the transgenic

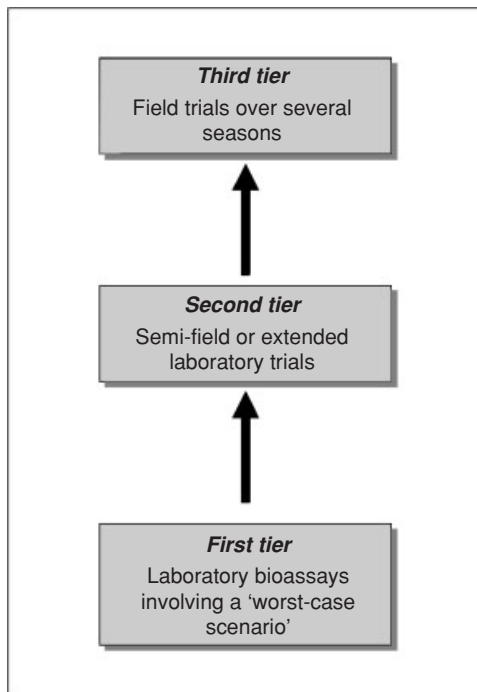


Figure 8.2 Three-tiered risk assessment.

product either by direct contact (typically trophically) with the introgressed recipient itself or in the rhizosphere.

The principal advantage of a tiered approach is that the gross effects of gene introgression could be determined by relatively simple bioassays. Such a system thus combines economics with environmental safety and allows a way forward that can combine these two, often opposing, factors. However, subtle direct effects and especially indirect effects can often be missed during first-tier tests, and so must be investigated at the population level in the higher tiers. An example of this would be to look at the population effects of a slight reduction in the fecundity of an individual female parasitoid after exposure to a host feeding on introgressed plant material in a first-tier study. The critical question relates to whether this has any effect on the population dynamics of these insects when more realistic spatial–temporal and environmental factors are introduced at higher tiers. If there is no observable effect on the population dynamics of the host and parasitoid, then it is unlikely to have any ecological significance.

The ultimate aim of the tiered risk assessment is to provide some quantitative measure of the risk of introgressed transgenes to non-target species. However, the tiered risk assessment does have limitations in this regard, in that it can only serve to identify qualitatively the magnitude of exceeding exposure, simply because of uncertainty and lack of precision. The outcome will serve as direction to return to the detailed, quantitative no-choice bioassays to pursue any necessary risk mitigation strategies. In other words, the tiered risk assessment may be regarded more like a barometric reading, hinting at wider scale change, which should be used in conjunction with a refined ‘weather forecast’ of the comprehensive assessment. This should provide a specific measure of the risk for transgenic crops and perhaps most importantly allow the public to judge the costs versus the benefits.

Increasing our knowledge of the behaviour and fate of transgenic plant proteins in the environment should enable the development of predictive models (see Section 8.8.2), calibrated by empirical data from both laboratory and field studies. Such modelling may be the best way forward for predicting the consequences of exposure to transgenes in introgressed recipients as this overcomes the potential limitations and inherent risks associated with field experiments.

8.5 Quantifying exposure with first- and second-tier experimentation

8.5.1 Exposure in non-target herbivores and pollinators

Crops producing *Bt* toxins have a relatively high degree of specificity depending upon the particular Cry protein expressed. The Cry1 and Cry2 groups of proteins are specific to Lepidoptera, while Cry3 toxins are specific to Coleoptera. Non-target organisms that could potentially consume transgenic plant material, should gene introgression occur, may include species from these two orders. In addition, insects from other non-target herbivorous orders, such as Hemiptera and Thysanoptera, may

also ingest toxins when feeding on transgenic plants, although this is dependent upon where in the plants the proteins are expressed.

The behaviour and fate of *Bt* toxins in non-target herbivores is largely unknown, although there are several published studies that have examined the impacts of *Bt* crop plants on non-target organisms. Hilbeck *et al.* (2000) reviewed the effect of *Bt* plants on non-target organisms, and suggested that experiments did not always accurately simulate the potential routes of exposure that may occur in the field; and as with pesticide ecotoxicology testing, selection of test organisms was not always conducted on ecologically relevant species. However, as it is not possible to test all scenarios with all non-target invertebrates, risk assessment must focus on a select few indicator species. Although not always ecologically relevant, they are often selected because they are highly sensitive to change and they are often good predictors of change in other more vulnerable species.

8.5.2 *Exposure in natural enemies*

Organisms at higher trophic levels such as predators and parasitoids can be either directly or indirectly exposed to proteins from transgenic plants. The effects of transgenes on natural enemies have been reviewed by Groot and Dicke (2002) and Poppy and Sutherland (2004).

In addition to taking prey items, natural enemies such as coccinellids may also be facultative feeders on honeydew excreted by aphids and on plant parts, especially pollen. Therefore, these insects could be directly exposed to transgenic proteins that might be present in an introgressed recipient. There is also a possibility of indirect effects of exposure to the toxin, for example there might be a reduction in the quality or numbers of prey or hosts that are available to the natural enemy, which in turn could alter the foraging behaviour of predator or parasitoid.

Investigating the effects of toxins on tritrophic interactions is highly complex, since the experimental protocols must simulate accurately the route of exposure to the toxin. A majority of laboratory studies have focused only on the potential direct toxicity of transgenic crop plants to natural enemies, and only now are we beginning to think about exposure to transgenes in a recipient plant. However, we should be able to draw inferences from effects observed in GM cultivars to the possible ecological consequences of the transgenic protein in an introgressed wild or weedy recipient.

Both direct and indirect effects of transgenic *Bt* plant material have been demonstrated experimentally. Direct toxicity in green lacewing, *Chrysoperla carnea*, larvae was reported when *Bt* toxins were incorporated into an artificial diet (Hilbeck *et al.*, 1998b). However, in the ladybird, *Coleomegilla maculata*, there were no direct negative effects of consuming pollen containing the Cry3Bb *Bt* toxin on the fecundity, pupal weight and developmental time (Pilcher *et al.*, 1997; Lundgren & Wiedenmann, 2002). Indirect effects on predators and parasitoids may also be observed because of a lower abundance or quality of hosts, e.g. when *C. carnea* larvae consumed *Bt*-fed *Spodoptera littoralis* and *O. nubilalis*, mortality was

always significantly higher than in controls (Hilbeck *et al.*, 1998a), although the validity of these studies is questionable as the lacewings were forced to consume a non-preferred prey item (Poppy, 2000). This represents a suboptimal diet that could create an undesirable outcome or cause a magnification of its effect. Additional work by the same group has shown that lacewings preferentially feed on aphids when offered a choice of insects (Meier & Hilbeck, 2001). Since the aphids were not ingesting the *Bt*, there would likely be no direct or indirect effect of *Bt* maize on lacewings in higher tier experiments or first-tier experiments that offer a choice of diets.

Insect physiology will also have a bearing on exposure to the transgene protein. For example, the aphid parasitoid, *Aphelinus abdominalis*, excretes most of the snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), ingested. Sublethal effects of GNA were also studied and no detrimental effect was observed. However, GNA had an indirect host-size-mediated effect on the sex ratio and the size of parasitoids developing in GNA-fed aphids (Couty & Poppy, 2001; Couty *et al.*, 2001), with more male parasitoids being produced from the GNA-fed aphids. This could, of course, alter parasitoid populations in the field. First-tier testing by Schuler *et al.* (1999b,c) showed that mortality of *Cotesia plutellae*, a parasitoid of the diamondback moth, *Plutella xylostella*, was almost 100% when developing on *Bt* oilseed rape. If inferences were drawn from this alone, then one would report that the transgenic plant affects the parasitoid directly. However, the parasitoids died because there was insufficient time to complete their development within *P. xylostella* larvae reared on *Bt* oilseed rape because their host died as a result of the toxin. However, when *Bt*-resistant diamondback moths were used in similar trials there was no difference between parasitoid mortality developing on *P. xylostella* feeding on wild-type oilseed rape and the GM oilseed rape (Schuler *et al.*, 1999c).

All of the above experiments demonstrate the potential power of first-tier bioassays in elucidating the importance of direct and indirect effects in determining cause and effect, and these will prove essential parameters to input into the risk assessment. They also demonstrate that by including appropriate biological factors into simplistic assays, much more powerful ideas can be developed for more detailed assessment in higher tiers.

By increasing the spatial and temporal complexity of the bioassays, second-tier studies invariably allow observations at the population level. They are typically conducted in an extended laboratory-type setting a glasshouse or an enclosed cage within a larger field. They offer an intermediate scale of risk assessment between worst-case scenario testing and full-scale field-testing. A good example of a second-tier experiment includes the work by Schuler *et al.* (2001), using mixtures of GM (expressing *Bt* CryIAc and the proteinase inhibitor oryzacystatin I) and wild-type oilseed rape plants in large field simulator cages. There were no differences in parasitism rates of the aphid, *Myzus persicae*, by the parasitoid, *Diaeretiella rapae*, on GM and untransformed plants. These types of exposure experiments represent an important component in the risk assessment process as they provide a link between simple bioassays and field-scale experimentation.

While we can draw inferences on the possible ecological effects and consequences of gene flow using crop cultivars, this will not be a substitute for the effects when introgression occurs, given the often marked differences in physiology of the wild relatives. Ideally, hybrid plants with introgressed transgenes should be used in first- and second-tier bioassays similar to all of the above examples to elucidate the effects of the transgenes, although this has not really been attempted until recently (Mason *et al.*, 2003).

8.5.3 *Exposure in soil organisms*

A great diversity of soil organisms could potentially be exposed to transgenic plant-derived toxins in the soil. Soil organisms could possibly be exposed directly via contact with the roots (whether trophically or not), exudation of toxins from roots into the rhizosphere and post-harvest incorporation of plant debris into the soil (Saxena *et al.*, 1999, 2002; Saxena & Stotzky, 2000). Soil organism exposure to transgenic proteins may also be investigated in relatively simple first- and second-tier bioassays. Rather surprisingly, there is a lack of quantitative data and published studies on the ecological effects and consequences of *Bt* present in the soil beneath transgenic plants, whether these are crop plants or wild or weedy recipients. However, some studies have been carried out in both cultivated maize and potatoes. Again, it is predicted that techniques used and information gleaned from the effects of GM crops on soil organisms would be directly transferable to the effects of transgenically expressing similar wild or weedy recipients.

First-tier soil exposure studies have been carried out by Saxena and Stotzky (2001) in which they found that root exudates from Cry1Ab maize showed no deleterious effects on bacteria, fungi, protozoa, nematodes or earthworms. Griffiths *et al.* (2000) also used laboratory studies to examine the impacts of exposure to transgenic potatoes expressing the genes for GNA and concanavalin A (Con A) on several non-target soil organisms. Again, they were unable to detect detrimental effects of the lectins on the bacteria and protozoa, although minor indirect effects were reported in a bacterial-feeding nematode in that its prey-location was reduced (Griffiths *et al.*, 2000). Other work has investigated exposure to transgenic proteins in higher invertebrates, and both Wandeler *et al.* (2002) and Escher *et al.* (2000) looked at the decomposition of *Bt* maize by the woodlouse, *Porcellio scaber*, with both groups uncovering conflicting findings.

8.6 **Quantifying exposure with third-tier experimentation**

The final tier in the risk assessment should be field trials, preferably conducted at a large scale over more than one season. Field studies may often be essential to quantify ecological exposure to hazards and thus estimate the risk posed to the environment by a crop × wild plant transgenic hybrid. Experiments in the field can be a very powerful means to predict ecological responses to changed conditions, but should ideally be

supported by lower tier investigations. While laboratory and greenhouse studies offer the possibility of close control over environmental variables, it is difficult, or impossible, to accurately replicate field conditions and therefore predict actual impacts on ecosystems.

The importance of scale in field experiments cannot be understated and there are obviously options to alter the scale at which processes take place. In general, larger plot size combined with higher numbers of replications will enable a wider range of environmental conditions to be studied and therefore give a greater degree of accuracy in measuring responses to transgene exposure. However, including too many variables in field experiments may mean that it is difficult to separate out the impacts of the transgenic traits being investigated. Therefore, the design of field experiments usually involves a compromise between the ability to control for environmental variation, the degree of accuracy required and also the cost of field research, which is a major consideration. Additionally, field research involves a deliberate release of transgenic hybrid plants into the environment and as such may involve greater risks of environmental impacts such as additional gene flow to other closely related recipients near trial sites.

Field experiments can be an excellent means for examining likely responses and effects, but can be expensive and highly contentious because of public opposition. They are most robust when they represent ecological conditions realistically. For example, for hybrid plants with an introgressed insect-resistance gene, much of the experimental research on impacts of plant-derived toxins on non-target organisms has been carried out only in the laboratory (see previous sections). Research at the field scale has been very limited, often because of regulatory restrictions. Laboratory research can be useful in identifying potential hazards or impacts of gene flow but these can only be tested reliably by realistic field-scale experiments.

8.6.1 *Exposure in herbivores and pollinators*

There is currently little published and peer-reviewed scientific information available on the ecological impacts of transgenic hybrids in the field, so again we must draw inferences from the effect of commercial GM crops on non-target herbivores. Most of the field-scale research has been carried out on small-scale plots in the United States where these crops are already commercialised. However, several other studies involving impacts on non-target organisms in *Bt* crops in Europe and China are now underway.

While not an example of gene flow *per se*, the monarch butterfly case study can again provide us with valuable information as to the possible consequences of gene flow from *Bt* maize to a wild recipient such as teosinte (*Zea* spp.). It had been reported that pollen from *Bt* maize (event 146) with high expression level could increase mortality in monarch butterfly larvae (Losey *et al.*, 1999; Hansen & Obrycki, 2000). However, a negative result from a laboratory study is not indicative of a real risk in the field. The laboratory research was based on a worst-case scenario that would be very unlikely to occur under natural conditions. An array of studies was

carried out specifically to test whether evidence of harm to a non-target herbivore demonstrated in the laboratory translated into a detrimental impact in the field (Sears *et al.*, 2001), involving a series of detailed assessments of both hazard and exposure.

The results did demonstrate that monarch larvae feeding on milkweed leaves in field plots of one variety of *Bt* maize (event 176), which contains high levels of Cry1Ab in the pollen, had 60% lower survival than in control plots. However, there were no significant negative impacts to monarch larvae in plots with other *Bt* maize cultivars (Stanley-Horn *et al.*, 2001). It was also found that 90% of pollen landed on vegetation within 5 m of maize plants and that most milkweed plants tend not to be found close to maize fields (Pleasants *et al.*, 2001). Although in separate studies, Chilcutt and Tabashnik (2004) found moderate levels of Cry1Ab in non-transgenic maize ears, some 31 m from the GM plants. Pleasants *et al.* (2001) also recognised that the presence of maize pollen and monarch migration does not coincide. Maize pollen is typically released during a 2-week period and the peak influx of migrating monarchs and release of maize pollen were found to overlap in between 15 and 62% of fields (Oberhauser *et al.*, 2001).

Overall, the studies show that event 176 *Bt* maize could have adverse effects on monarch butterflies in the field, although exposure was likely to be limited. In all other varieties under investigation, there were little or no impacts on monarch populations. The studies did not examine monarch population dynamics at the field scale throughout a whole season, so there is a possibility that the less toxic *Bt* varieties could have chronic sublethal effects on monarchs, although the overall impacts on populations would still probably be low or negligible (Sears *et al.*, 2001). Other factors, especially conventional agricultural activities, are likely to have a far more significant effect on monarch population dynamics.

This is now *the* classic example of an exposure study that focuses on a particular species or group of species, rather than the agroecosystem as a whole. It is essential to test specific hypotheses when potential hazards and exposure have been identified, but regrettably these studies can tell us little about the overall impact on overall biodiversity, should 176 *Bt* maize hybridise with its wild relative.

The most well known example of a third-tier study that assessed the ecological consequences of transgenic plants on biodiversity was the FSEs of GM herbicide-tolerant (GM HT) crops in the United Kingdom (Firbank *et al.*, 1999, 2003). This investigation focused on the impacts of change of management practice on farmland biodiversity when growing GM HT beet, maize and oilseed rape. The monitoring and assessment of invertebrates was an essential component of the sampling as they were used as measures of ecosystem functioning, keystone species, biomass for feeding higher trophic levels and indicators of environmental change. This illustrates the importance of invertebrates, in particular insects, in assessing the environmental impacts of transgenic plants, even if the plant has not been engineered to affect insects directly. From data collected over 4 years in the FSEs, in general, weed biomass was reduced under GM HT management in both beet and oilseed rape but increased in maize compared with conventional treatments. Not surprisingly, changes in weed resource had significant effects on some organisms at the second

trophic levels because of the decreased abundance in food. However, most of the higher taxa studied were insensitive to differences between GMHT and conventional weed management (Haughton *et al.*, 2003).

Possibly the most important product of the FSEs is that they demonstrated that invertebrate groups in agroecosystems are highly sensitive to changes in weed communities arising from altering management regimes whether these be novel herbicides or GM crops (Hawes *et al.*, 2003).

8.6.2 *Exposure in natural enemies*

Several field studies on exposure of transgenic plant products to beneficial insects have been performed. These assessed the potential impact of commercial *Bt* crops on several natural enemies, including coccinellids, chrysopids and anthocorids, but again this work is of direct relevance to wild relatives that may express the same transgene. There were no significant differences in the overall density of beneficial insect populations between *Bt* and non-*Bt* maize (Pilcher *et al.*, 1997). However, it should be noted that these were small-scale studies with plot sizes of just 45 m² with just three replications. A more robust field study, as the plot sizes were much larger, was the research by Orr and Landis (1997). They found no effect of the transgene protein on natural enemy populations, although they were guilty of ‘snapshot sampling’ (Crawley, 1999; Poppy, 2000; Poppy, 2004) as natural enemies were recorded on just three days in late summer. However, in the investigation by Wold *et al.* (2001) there was a trend for non-*Bt* treatments to have a higher number of ladybird, *C. maculata*, larvae than in *Bt* maize. However, the authors do add that this may be due to relatively subtle population effects and recommend further research with larger sample sizes and spatial scales to investigate predator population effects of *Bt* maize (Wold *et al.*, 2001). Wilson *et al.* (1992) also provided field evidence that *Bt* cotton has no effect on chrysopids. For many of these studies, sampling of beneficial insects was often incidental to recording effects on pest species and samples taken only three or four times in a season. It will be increasingly important when looking for the ecological effects of transgenic plants, be they wild recipients or cultivated varieties, on non-target organisms that we do not rely upon incidental or snapshot sampling (Crawley, 1999) as using these data will undoubtedly diminish the power of the risk assessment. It is also important that field studies are conducted as part of a tiered risk assessment framework to ensure the correct questions are asked and the focus is appropriate with adequate trigger values and thresholds for action.

8.6.3 *Exposure in soil organisms*

Assessing the possible *in situ* effects of exposure to transgenic proteins in soil organisms is fraught with many problems and confounding factors. Consequently, very few investigations have focused their attention on the effects of transgenic plants on soil organisms and those that have been done were with commercial crop varieties. A transgenic nematode-resistant potato plant expressing cysteine

proteinase inhibitors was used in a study by Cowgill *et al.* (2002) to test the effects on soil organisms. The transgenic lines had no significant effect on the abundance of soil microarthropods or nematodes. In the second year of the investigation, microbial abundance in transgenic lines was reduced relative to the controls, although these observed reductions had no effect on the rates of litter decomposition (Cowgill *et al.*, 2002).

In the earlier mentioned study, Griffiths *et al.* (2000) conducted field studies with potatoes expressing Con A and GNA lectins. They found that GNA-containing potatoes significantly changed the physiological profile of the rhizosphere community at harvest but effects did not persist into the next season and had no effects on the growth of a subsequent barley crop.

The FSEs also monitored effects of GM HT crops on the soil surface fauna, notably the Collembola, and did show some significant effects. However, no significant treatment effect was recorded for total collembolan catches in any of the three FSE crops. There were within-year effects, and counts of total Collembola were consistently greater in the GM HT treatment in August in beet and maize and in July in spring oilseed rape (Brooks *et al.*, 2003).

To summarise, studies on the impacts of transgenic plants on soil processes have shown some minor detrimental effects in soil microbial community structure, but to date there is no concrete evidence to demonstrate that this could adversely affect soil health in the long term. The minor effects that transgenic plants exert on soil microbial ecology are pale in comparison with the typical sources of variation experienced in agroecosystems and associated habitats (Kowalchuk *et al.*, 2003).

8.7 Planned releases of GM crops

Small-scale field experiments are unlikely to detect all possible environmental effects of transgenic crops and associated risks of gene flow. As we have seen in preceding sections of this chapter, there has been contradictory evidence as a result of field trials influenced by normal environmental variation. In general, even small-scale field trials may only be sensitive enough to detect extremely large effects. For any such series of experiments, there will be bounds to what can be detected and these limits will be high because of the large variation from one experimental unit to another. The FSEs required detailed power analyses to ensure that there was confidence in detecting significant differences, and thus the public, regulators and scientists could have faith in the conclusions drawn by these comprehensive trials. It is therefore a requirement (certainly under EU law) to conduct post-commercialisation testing to determine if the tiered testing protocols adequately assessed exposure. This is not unusual, as quality control is put in place for most, if not all, novel technologies. It is also equally important to establish long-term, monitoring programmes to record trends in predicted effects and to detect effects that were not predicted by pre-commercialisation testing. However, if taken to extremes, post-release testing

and monitoring is likely to be prohibitively expensive, and if not carefully conceived could lead to collection of highly spurious and non-interpretable data.

The three most important facets of post-release environmental monitoring for transgenic crops and possible gene flow are to first evaluate the actual need for and approaches to environmental monitoring, and if deemed necessary to include recommendations for post-release monitoring of transgenic plants. Second, guidance should be provided on the assessment of non-target exposure effects including appropriate tests for environmental evaluation. Finally, assessments of the cumulative effects on both agricultural and non-agricultural environments for transgenic plants should be conducted, whether for crop plants or for possible introgressed recipient species.

Undoubtedly, the most important question to ask when considering post-release monitoring is 'will exposure to the transgene protein or product increase in non-target organisms as a result of persistence or spread of the transgene in the environment?' The PROSAMO (planned release of selected and modified organisms) programme sought to address this question and studied the persistence in the environment of four different GM crops (GM HT oilseed rape, maize and sugar beet and *Bt* potato) (Crawley *et al.*, 1993, 2001). In the 12 types of natural or semi-natural habitats under investigation, GM HT maize never persisted for more than 1 year and the longest-lived sugar beet was just 2 years (Crawley *et al.*, 2001). GMHT plants of oilseed rape and sugar beet produced second-generation plants in 1 or more of the 12 sites, but GM insect-resistant potato and HT maize did not. The evidence suggests that we should assume that at least some GM crops would produce second-generation plants following escape from agriculture, at least in some habitats. The key point is the amount of second-generation plants produced, whether directly or via hybridisation with wild relatives. In the PROSAMO experiments, none of the GM oilseed rape, sugar beet, potato or maize plants increased in abundance in any of the sites (Crawley *et al.*, 2001). All of the transgenic plants (and their conventional counterparts) declined to extinction within 1–4 years. In all cases, failure to pass the invasion criterion was due to the combined effects of plant competition and herbivory. Thus, while it is possible in principle for transgenic plants to increase in abundance following escape from arable cultivation, the evidence suggests that this will not occur in any of the habitats so far investigated for the GM crops currently available. While Crawley's study looked at transgenic crop plants, these results have obvious significance when considering the ecological implications of an introgressed hybrid plant containing a transgene.

In principle, however, transgenes that can confer a clear fitness advantage to a GM plant, e.g. insect or virus resistance, rather than simply herbicide tolerance do have the potential to establish and persist outside of arable fields. Such traits require case-by-case field-testing for invasiveness and it would be very unwise to generalise from GM HT plants to all other transgene constructs. However, the current scientific consensus is that at present there is no evidence that current commercial GM crops would be more invasive than their non-GM counterparts would be if released into the environment. However, in the future, transgenic plants may not be comparable

with non-GM plants because transgenic technology may have the ability to change fundamentally the physical and reproductive architecture of plants to the point where they could effectively become new species, and thus act more like an exotic plant introduction (e.g. *Rhododendron*). Such exotic plants with novel genomes may pose more of a risk than endemic or widely grown plants containing only a few novel genes in their genome (M. Crawley, personal communication, 2004). Only field-testing is likely to provide definitive answers to these questions.

Examining the occurrence and actual consequences of transgene introgression in wild relatives under field conditions may prove to be an essential tool in the risk assessment process. The approach may be either to examine the experience from growing the same or similar varieties elsewhere (e.g. in North America) or monitoring the ecological consequences of GM crops post-release. Examining the consequences of the same or similar varieties grown elsewhere has the advantage that the consequences of realistic, and sometimes large-scale, planting can be assessed before the crop is actually introduced. Although likely to produce useful insights, there is the issue that agroecosystems and wild recipient species themselves vary between countries and continents, and so there is the likelihood that responses will differ. EU risk assessment also requires that field trials must be conducted in European environments or that adequate bridging studies be carried out (EU Directive 2001/18/EC). Comparison with experience elsewhere is obviously a very useful approach but there has been surprisingly little work studying existing commercially grown GM crops, probably because farmland biodiversity does not have the same significance in the countries where GM crops are currently commercialised. However, if there were dramatic effects of transgene introgression into wild relatives of crop cultivars, then it seems probable that these would have been detected in countries where transgenic crops have been cultivated for the last decade.

8.8 Alternative approaches to quantifying exposure

8.8.1 Simulation studies

Where it is not possible to grow plants containing introgressed transgenes under actual field conditions because of local or regional restrictions, it might be preferable to mimic the action of the transgene. For example, the action of transgenes conferring insect resistance could be mimicked by the targeted application of selective insecticides, although care is needed with regard to the choice of insecticide (Schuler *et al.*, 1999a), the time of application (Riggin-Bucci & Gould, 1997) and the manner of application. Ultimately, simulation could be a more useful tool for hazard identification than for exposure quantification.

At the time of writing, in the United Kingdom and across much of the European Union, the most tractable method for quantifying the consequences of exposure to transgenes is by controlled experimentation using substances to mimic the presence

of a gene rather than controlled a priori releases of transgenic material, which is severely restricted under UK and EU law. This is particularly the case for simulating insect-resistant crops such as *Bt*, which will be described herein. It may also be possible to simulate the ecological consequences of introgression of a fungal disease-resistance transgene by application of fungicides to mimic the action of the transgene. We will now attempt to answer the question as to whether microbial *Bt* sprays can effectively be used to simulate the effects of hybrid plants expressing a *Bt* transgene and outline some of the limitations of simulating insect resistance.

8.8.1.1 *Advantages of transgene simulation*

Current commercial insect-resistant transgenic plants rely solely upon the production of toxins derived from *Bt* and because of *Bt*'s high degree of pest specificity are only resistant to a limited number of herbivorous insects (Schuler *et al.*, 1998). *Bt* has been widely used as a lepidopteran biopesticide since the 1950s (Navon, 2000), and so there is already a great wealth of information available on microbial sprays, particularly concerning its ecotoxicology and effects on non-target organisms. For example, several studies have reported that microbial *Bt* has a detrimental effect on non-target Lepidoptera (Miller, 1990; James *et al.*, 1993). This ecotoxicology data is particularly valuable when considering the interactions at higher trophic levels and it has been demonstrated that microbial *Bt* does have detrimental effects on higher trophic levels (Horn, 1983). Effects on a diverse range of other non-target species have also been widely demonstrated (e.g. Bellocq *et al.*, 1992). It is vitally important not to disregard the some 50 years of data on *Bt* as it can teach us a great deal about the possible ecological consequences of the presence of *Bt* transgenes when expressed in crop plants or wild recipients.

8.8.1.2 *Disadvantages of transgene simulation*

There are many problems associated with attempting to draw inferences from plants treated with a microbial *Bt* spray and extrapolating that to the ecological consequences of a hybrid plant expressing a *Bt* gene. First, a microbial *Bt* spray usually contains a variable complex of several different δ -endotoxins, while current transgenic plants tend to have only one gene expressing a truncated δ -endotoxin. For example, Dipel, the most widely used microbial *Bt* formulation, contains five crystal proteins (Glare & O'Callaghan, 2000). Microbial formulations containing multiple toxins tend to be more effective than an encapsulated single crystal toxin in killing their target (Asano & Seki, 1994). However, the newer generation transgenic crops have pyramided genes encoding for several Cry proteins to limit resistance development, and so it may be possible to make comparisons between these and the more pure microbial formulations.

Microbial *Bt* sprays are typically applied as a spore formulation and some workers have suggested that the spores act in synergism with the endotoxins and markedly enhance their efficacy (Dubois & Dean, 1995). Most studies, however, have shown that the spores play only a minor role in increasing insect mortality. A more important confounding factor is that microbial sprays also contain other formulation products

such as gum arabic, lactose and silica, which are added to improve the environmental stability of the endotoxins. These additional components could have a profound effect on insect behaviour, as found in investigations with microbial *Bt* where it was the surfactants present in the formulation that markedly increased oviposition by *P. xylostella* (Riggin-Bucci & Gould, 1996).

Despite the presence of these formulation components, which are often added to increase the longevity of the microbial *Bt* on the plant, sprays still have a limited persistence on foliage (Fuxa, 1989). This decline in persistence would mean that a plant's protection from herbivory would decline with time. In a transgenic plant, endotoxins are continually expressed and so present a continuous selection pressure on insect herbivores. This persistence of the microbial compound is affected by a wide range of abiotic factors, which are discussed in detail by Glare and O'Callaghan (2000), but the principal environmental conditions affecting persistence are rainfall and/or dew, UV light and temperature.

Another major constraint of simulating the presence of a transgene with a microbial spray is the inability to protect inaccessible plant tissues (Ely, 1993), while most transgenic plants constitutively express the endotoxin and so protect endogenously. The majority of transgenic plants use the 35S promoter for constitutive expression, although there is an increasing number of tissue-specific promoters that can select where the protein is produced. Delannay *et al.* (1989) made comparisons of transgenic and microbial *Bt*-treated tomatoes and found that numbers of the tomato pinworm, *Keiferia lycopersicella*, which feeds internally, were significantly reduced by the transgenic plant but not by the microbial spray. Similarly, there is also the issue of placement of microbial sprays. Many insect herbivores, such as *P. xylostella*, feed on the undersides of leaves, but microbial sprays would most likely be applied to upper leaf surfaces unless they are applied under an electrostatic charge (A. Dutton, personal communication, 2001).

There is also a major difference in the activation of toxins between microbial sprays and transgenic plants. In a microbial spray, a high pH in the insect gut is essential to cleave the protoxin and thus activate it. In *Bt* plants, the toxin is present in a truncated form, which is already partially activated and does not require a high gut pH. This truncated toxin is a significantly smaller molecule than the protoxin produced by the bacterium. It has been hypothesised that truncation may alter the host specificity spectrum, creating novel non-target risks dissimilar from microbial *Bt* insecticides (Jepson *et al.*, 1994; Hilbeck, 2001; but see MacIntosh *et al.*, 1990). Therefore, transgenic plants would most likely have a wider range of activity than that delivered by a microbial spray (Dutton *et al.*, 2002). Hence, more robust assessment techniques must be developed that account for these differences (Jepson *et al.*, 1994). As suggested by Jepson *et al.* extended dietary exposure bioassays of non-target organisms would be one logical approach towards this goal. All the evidence to date, however, suggests that the truncated *Bt* toxins in transgenic plants do not lead to changes in specificity compared to microbially sprayed *Bt*.

In addition, *Bt* toxins remain in the dead transgenic plant tissue after senescence. As we have seen earlier in *Bt* maize, the Cry toxins can enter the soil via root

exudates (Saxena *et al.*, 1999), thus increasing exposure in soil organisms. The activated toxin can bind to clay particles and retain insecticidal activity for more than 9 months (Saxena *et al.*, 1999). The protoxins in *Bt* insecticides do not readily bind to clay and degrade rapidly in soil. Consequently, the toxins from *Bt* maize have several unique exposure pathways not characteristic of the microbial spray.

8.8.1.3 Future scope of transgene simulation

As far as we are aware, simulation studies using microbial *Bt* are rare. No research was found in the published literature that directly examines the ecological impacts of a transgenically expressed *Bt* relative to microbial *Bt* and/or routine chemical insecticide applications. The only comparable field study is the work of Reed *et al.* (2001). In this investigation, control of the Colorado potato beetle, *L. decemlineata*, and the effects on non-target insects were compared under both transgenic Cry3Aa *Bt* potatoes and sprays of permethrin and microbial *Bt*. The transgenic potato provided significantly better control of the pest and also supported more of the generalist predators, *Geocoris* sp., *Nabis* sp. and *Orius* sp. Delannay *et al.* (1989) also made comparisons of transgenic and microbial *Bt*-treated tomatoes; however, this was for an internally feeding herbivore and it is clear that microbial *Bt* would underperform in this example. Possibly, Tabashnik *et al.* (1992) are the only workers to have used mortality data from both foliar treatments of microbial *Bt* and transgenic plants to calibrate simulation models.

Similar experimental protocols could be employed to investigate possible effects of gene introgression by application of microbial *Bt* to hybrid plants that might be formed during introgression, although the caveats mentioned earlier must be recognised to avoid spurious conclusions.

8.8.2 Modelling

Under current financial restrictions, most field studies typically last 2–3 years, which is problematic when trying to predict the long-term effects and consequences of gene flow from transgenic plants. However, should the experimental approach outlined in previous sections highlight minor detrimental ecological effects of gene flow from GM crops, then modelling could be used to predict both their wider scale and also longer term implications. It may be possible to predict the likelihood and long-term consequences of exposure to transgene proteins using modelling. Models are simply a mathematical description of several plausible scenarios and provide a rigorous understanding of what might and might not occur in nature. Models have the considerable advantage that they can make use of pre-existing databases and knowledge, and by the application of information regarding the underlying processes, it may be possible to predict the responses of an agroecosystem to a novel situation such as a gene flow event (Messean *et al.*, 2003).

The most basic models are population-type models employing a series of equations to describe the ecological interactions using fecundity, mortality and movement

rates and how these are affected by population density. It is then possible to predict what happens to the expected population if one or all of the above parameters are altered. The scenario could be modelled as a single population, a metapopulation or a cell model (Tilman *et al.*, 1997). A single population model would only be useful if the population were closed, i.e. all recruitment occurs from within the population. In such a case, a dynamic model could be developed with two genetically distinct subpopulations comparing the transgenic wild relatives and non-transgenic plants using demographic information such as births, deaths and immigration and emigration between the subpopulations (Thompson *et al.*, 2003). A metapopulation model may be appropriate when several interacting populations are spatially separated but may have exchange of individuals. Such models employ single-population modelling techniques but with additional immigration and emigration between the populations of the metapopulation.

Finally, the use of a cell model may be valid when the detailed distribution of organisms in a specific area is desired. Cell models have found a variety of applications in ecology where knowledge of the spatial position of an organism is important, such as the location of an insect on an individual plant or in a field. Simple models would allow the tracking of local movements into adjacent cells with given probabilities.

When modelling the ecological impact of a specific transgene in the environment we can use exposure–dose–response models. These establish specific endpoints (Poppy, 2003), which are the values that we are trying to protect by undertaking a risk assessment. For example, in evaluating the risk posed by planting *Bt* oilseed rape and the hypothetical introgression of the transgene into a wild species such as *Brassica rapa*, we may be concerned about the ecological impacts that it may have on rare, endangered or other non-target insects. For example, both the green-veined white (*Pieris napi*) and the orange-tip (*Anthocharis cardamines*) are known to feed on *B. rapa*, and therefore populations of both species and their natural enemies could be affected should the caterpillars consume the hybrid plant expressing *Bt*. This is described in Figure 8.3. Thus, our assessment endpoint may be defined as the point at which there is no decrease in the population of the herbivores and their natural enemies as a result of gene introgression. Such an evaluation involves an exposure–dose–response assessment. The exposure assessment calculates the likelihood of exposure, the dose assessment estimates the actual amount of toxin larvae are exposed to and the response measures the physiological reaction to the dose. The dose received then depends on the amount of leaf material consumed and the concentration and toxicity of the *Bt* Cry proteins within the plant. The likely mortality in the population model may be estimated by reference to a dose–response curve derived from relatively simplistic laboratory experiments, such as those described in Section 8.5.

Possibly, the most significant application of modelling to determine the ecological consequences of transgenic plants is the model developed by Watkinson *et al.* (2000). The model was used to predict long-term changes in weed and bird populations

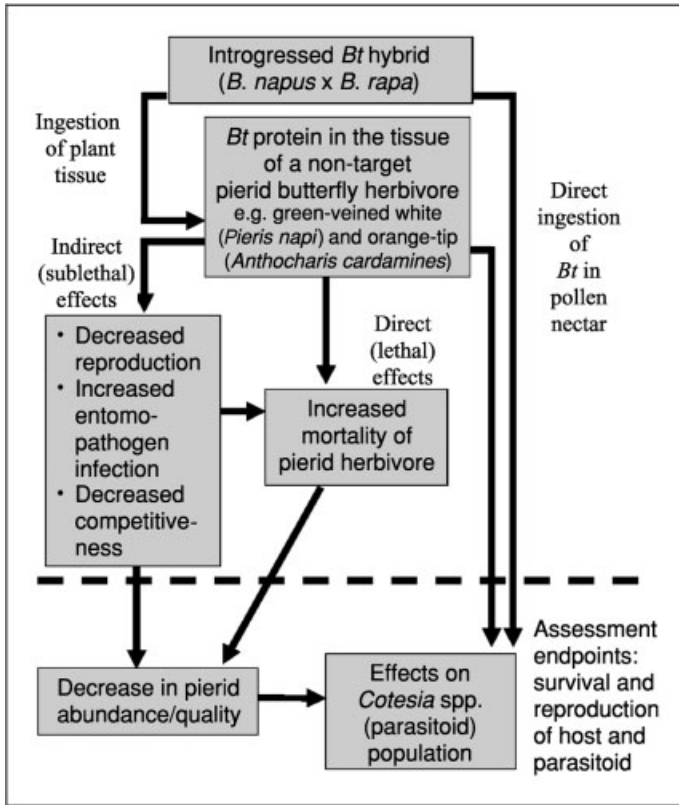


Figure 8.3 A conceptual model for assessing the impacts of an introgressed *Bt* gene in *Brassica rapa* on two non-target pierid butterfly herbivores (the green-veined white, *Pieris napi*, and the orange-tip, *Anthocharis cardamines*) and their parasitoid (*Cotesia* spp.) natural enemies.

rather than the response over just 1–2 years and predicted that GM HT crops could turn agroecosystems into faunal deserts, thus having a severely detrimental knock-on effect on bird populations. Although the modelled response was more a consequence of herbicide use than exposure to the transgene protein, models have a high plasticity and could be used to determine the effects of an introgressed transgene. Data from the FSEs should also provide essential empirical data that can be incorporated into population models such as Watkinson's in order to predict the long-term changes rather than the response over just 4 years (Perry *et al.*, 2003) so that landscape-scale predictions can be made concerning the exposure implications for a diverse range of taxa. However, in many taxa, there is such a paucity of available data that there is not yet the scientific background to use this approach with confidence. Ideally, modelling should generate new hypotheses for testing, which in turn lead to further

field experiments and collection of empirical data and refinement of the model (Crawley, 1999).

8.9 Conclusions

Risk assessment for GM crops and the consequences of transgene introgression into wild recipient species is currently entering a new phase. The potential increase in the number of GM cultivars will create the need for an integrated approach to risk assessment that is robust enough to withstand scientific and public scrutiny. Exposure trees permit case-by-case risk assessments that focus on hazards that have serious possible ecological consequences and a high likelihood of realisation. This new technology will not just bring new opportunities but will undoubtedly raise new risks and concerns about the role of agriculture in the environment. It is vital that the environmental risk of gene flow is fully assessed using the tiered risk assessment outlined in this chapter so as to reduce the potential for disruption to ecological systems. By providing a more accurate measure of risk, the benefits of GM crops can be considered against the risks and comparison made with alternative pest management strategies such as synthetic insecticides or the use of organic control measures.

The need for quantitative estimates of exposure is essential to feed into event-tree analysis exposure trees. Where widespread transgene spread is highly likely, attention must turn to fault-tree analysis and we need to work backwards from the hazard using a tiered risk assessment approach. This two-pronged approach provides a mechanism by which the gridlock referred to by Wilkinson *et al.* (2003) can be avoided.

The final challenge will be to accept that science is only one part of the equation that includes both moral and ethical issues. Science, however, should be at the hub of discussions providing the accurate information essential for informed debate and dialogue. Although it is sometimes easier to adopt a simple definition of the precautionary principle in order to stop scientific advances, decisions about scientific advances are necessary to assess the wisdom of maintaining the present system of agricultural production. Scientists should enter this new century with excitement about what can be done but should also be responsible about how these opportunities are used.

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9 Regulating the risks of gene flow

Steven Hill

9.1 Introduction

GM crops represent perhaps the most closely scrutinised agricultural technology there has ever been. Following a North American lead in the late 1980s and early 1990s, throughout the world regulatory regimes have been developed that seek to protect human health and the environment from perceived or actual risks posed by the cultivation of GM crops. The development of regulatory regimes builds on the realisation that, in some regards at least, GM organisms are special. But it also inevitably focuses attention on potential risks associated with GM crops that apply equally to crops developed using the less controversial techniques of so-called ‘conventional’¹ plant breeding. A prime example of this phenomenon is the subject of this volume – the movement and fixation of genes from GM crops into other varieties of the same crop, or other sexually compatible weedy and wild relatives. Genetic interchange of this type, facilitated by human intervention, has occurred since the beginning of agriculture, but only with the advent of GM technology has it received such detailed scrutiny and been the subject of a risk-based regulatory approach.

Within this context, my aim in this chapter is to discuss the regulation of GM crops in general, with particular focus on how potential risks associated with gene flow are considered within the regulatory system. I will concentrate on the European regulatory regime, although some of the principles that apply are common to many regulatory systems around the world, and there is a developing international consensus on environmental risk assessment (for example, the Cartagena Protocol to the International Convention on Biological Diversity²). In particular, I want to address the question of the interaction between scientific study of gene flow and the regulatory process; what information do regulators need to assess the potential risks associated with gene flow, and how can scientists best provide that information?

¹ ‘Conventional’ plant breeding encompasses not only the production of new varieties by crosses with sexually compatible relatives but also the use of a variety of other techniques including facilitated wide crossing, embryo rescue and mutation breeding.

² See <http://www.biodiv.org/biosafety/> for further information.

9.2 The European Regulatory Framework for GM crops

9.2.1 *Ensuring consumer safety: Directive 2001/18/EC and Regulation 1829/2003/EC on GM food and feed*

Legislation aimed at facilitating the use of GMOs for research and commercial use has been in place in the European Union (EU) since 1990. Two directives were agreed on in that year: 90/219/EEC on the Contained Use of GMOs (European Commission, 1990a) and 90/220/EEC on the Deliberate Release of GMOs (which includes commercial uses) (European Commission, 1990b). Directive 90/220/EEC was replaced in 2001 by Directive 2001/18/EC (European Commission, 2001). This legislation entered into force on October 17, 2002, and remains the central piece of community legislation in this area. The directive covers both the release of GMOs into the environment for the purposes of research, and their use as or in products that are placed on the market. The remainder of this discussion will focus on the regulation of commercial uses of GMOs. The basic provision of 2001/18 is that products containing or consisting of GMOs can only be placed on the market within the EU if they have specific, written consent obtained in advance under the provisions of Part C of the Directive – so-called ‘Part C consent’. The provisions of 2001/18 also require that separate permission is required before GMOs can be used in food destined for human consumption. Prior to April 18, 2004, approval for human food use was required under Regulation 258/97 on Novel Foods (European Commission, 1997). In 2003, however, new EU legislation concerning food and animal feed uses of GMOs was agreed on. Regulation 1829/2003 (European Commission, 2003a) became operational on April 18, 2004, and provides for a new centralised procedure for the authorisation of GM crops, food and feed. If permission is sought for the cultivation of a GM crop for use as food and feed, this can now be obtained solely through consent under Regulation 1829/2003 without necessarily obtaining Part C consent under Directive 2001/18.

Given that written consent is required for the placing on the market of a GM crop (including for cultivation), this raises the issue of the criteria that are used in deciding whether consent is given. Irrespective of the legislative route that is taken, the essential requirement is that there should be no adverse effect on human health or the environment resulting from the release of the GM crop. For example, Article 4(2) of 2001/18 states that

Member States shall, in accordance with the precautionary principle, ensure that all appropriate measures are taken to avoid adverse effects on human health and the environment which might arise from the deliberate release or the placing on the market of GMOs.

Similar measures apply to the new regulation of GM food and feed. At face value, this is a rather broad requirement to avoid adverse effects. However, the legislation elaborates how adverse effects should be identified – by carrying out an ERA. This is further refined by Annex II to Directive 2001/18, which sets out

important principles concerning the ERA, identifying the areas of potential hazard that should be considered and what elements of exposure are important in carrying out the ERA. Thus from the point of view of gene flow, central to the consideration of risks are:

- An assessment of the rates of gene flow between GM crops and wild relatives. Consideration will need to be given not only to the relevant cross-pollination rates but also to the extent to which wild relatives and crops flower at the same time (the exposure component of the risk equation).
- The potential for transferred genetic material to cause an adverse effect in the new genetic background (the hazard component of the risk equation).

The relationship between risk, hazard and exposure will be discussed further later.

A second central principle underlying the current application of the ERA for releases of GM crops is that this assessment is comparative. Risk is not considered in absolute terms, but by making a comparison with a suitable baseline. In most cases, the baseline used is the equivalent non-GM crop. This approach has significant implications. While there is some merit in the approach, it has the disadvantage that conventional non-GM practice may change and therefore the baseline against which GM crops are compared should also change. For example, herbicide-tolerant (HT) turf grass is being developed using both GM and conventional breeding strategies. From the perspective of gene flow of the HT trait, it could be argued that if the non-GM HT grass was widely marketed, the ERA for the GM variety conferring tolerance to the same herbicide only needs to consider novel aspects of the GM HT trait and this would not include a consideration of gene flow issues. A further weakness of the comparative assessment approach is that it may permit use of a GM crop that causes harm on the grounds that the equivalent non-GM crop also causes harm. A more rational approach would consider desired environmental targets, and regulate crop usage and management (in general, not just GM) to achieve those targets. However, in the current regulatory position there is a considerably greater possibility to regulate GM rather than non-GM crops, and so the comparative approach seems the only pragmatic way forward.

A further important feature of the approach to the ERA of GM crops is that only risks and not benefits are considered. Strictly speaking, if the ERA suggests that there is a significantly greater risk associated with a GM crop compared to its conventional counterpart, then authorisation should not be granted, irrespective of any benefits the GM crop may offer.

9.2.2 Ensuring consumer choice: Regulation 1830/2003/EC on traceability, labelling and coexistence measures

In addition to regulatory instruments whose purpose is to ensure the safety of GM crops, the EU has introduced further legislation with the aim of delivering consumer choice. The first labelling provisions were introduced in the late 1990s, but, in 2003, labelling of GMOs and products derived from them was firmly enshrined in

EU law through the adoption of Regulation 1830/2003 on traceability and labelling (European Commission, 2003b). This legislation requires that all material intentionally containing GMOs or products derived from them must be labelled, thus creating dual commodity streams within the EU. Given current consumer attitudes to GM food, this is likely to result in a price differential between GM and non-GM products. This in turn introduces significant negative economic implications for producers of non-GM crops should their crops contain detectable quantities of GM material.

Two provisions in Regulation 1829/2003 on GM food and feed are designed to deal with the possible economic repercussions for producers of non-GM food, feed and crops.

- The regulation establishes thresholds for the adventitious or technically unavoidable presence of GMOs in unlabelled material. These thresholds are 0.9% for GM material that has been authorised in the EU, and 0.5% for GMOs that have largely completed the authorisation process³. In the latter case this threshold is a temporary, transitional measure.
- Through the amendment of Directive 2001/18/EC, EU member states are given the power to introduce measures to ensure coexistence between GM and non-GM crops.

Importantly, the labelling provisions and the associated thresholds apply both to food and feed material containing GMOs, and also products derived from GMOs, such as highly refined oils and starch that contain, at least in the case of current GM crops, no detectable DNA or protein associated with the genetic modification and are not distinguishable chemically from non-GM material. From the perspective of our understanding of gene flow, the main implication of this legislation lies with measures to ensure coexistence between GM and non-GM crops.

Coexistence refers to measures designed to ensure that GM and non-GM crops can be cultivated freely so that neither approach is prevented. In practice, given consumer attitudes, the coexistence regimes that are being developed across Europe focus primarily on preventing the presence of GM material in the material harvested from farms where GM varieties have not been deliberately sown (including organic farms). In general, coexistence refers specifically to measures taken 'on farm' – delivering separate GM and non-GM food and feed supplies also requires segregated supply chains beyond the farm gate, but it is reasonable to assume that these can be delivered by the normal contractual arrangements found within the food industry. However, ensuring separation of crops at the farm level requires cooperation and, potentially, contractual arrangements between farmers. It is this goal that coexistence measures aim to deliver.

Coexistence regimes are likely to include a range of measures. There will be a need to ensure that GM presence within non-GM seed stocks is kept within limits, and further EU legislation in this area is expected. A range of measures are also

³ To qualify for this threshold the GMO must have a favourable opinion from the GMO panel of the European Food Safety Authority and meet other criteria, including the existence of a publicly available transformation-event-specific detection method.

required, particularly if both GM and non-GM varieties are cultivated on the same holding or handled with the same machinery. A further and essential feature of a coexistence regime for many crops will be the management of pollen-mediated gene flow, as this represents a mechanism whereby the genetic material contained in GM crops can be transferred to nearby non-GM varieties (see Chapter 3 in this volume). Designing such management measures requires a detailed understanding of the reproductive biology of the crops concerned, and in particular, of the factors that determine the extent of outcrossing and how cross-pollination rates vary with distance. For many crops, a large quantity of robust and reliable data already exist that allow gene flow management regimes based around separation distances and barrier crops to be developed. There is already a wealth of knowledge and experience in this area – approaches to isolation in seed production, the cultivation of conventional crops for industrial uses and the management of GM crop research trials all require this issue to be considered. While the detailed arrangements will need to be developed on a crop-specific basis, there are some important generic points. First, because of the nature of the relationship between cross-pollination rates and distance, it is not possible to completely prevent gene flow through the use of separation or barrier crops. Whatever measures are designed, it is necessary to decide, in advance, the maximum GM presence that will be tolerated in neighbouring crops – zero is not an option. Second, an important variable in determining the rate of gene flow is the size, both locally and at a landscape scale, of the pollen source (see Chapter 3). If there is increasing commercial uptake of freely outcrossing GM crops, maintaining non-GM versions below a given threshold will become increasingly challenging and require greater separation or wider barriers. Finally, it will be necessary to consider the extent to which wild relatives of crop plants may act as reservoirs of GM material that will influence the rate at which such genes appear in non-GM crops. While there is a good understanding of the rates of outcrossing between crops and their wild relatives, less is known about the extent to which introgression into wild populations occurs and, potentially, genes return to the crop species.

9.2.3 *The international context*

In contrast to the EU, neither the United States nor Canada has specific legislation aimed at regulating the commercial use of GMOs, but regulate GMOs using legislative instruments aimed at protecting human health and the environment from risks in general, rather than identifying GMOs as having the potential to be inherently risky. In the Canadian system, this concept is taken furthest in that the regulatory process does not single out GM crops, but rather focuses on ‘plants with novel traits’⁴. This means, for example, that in Canada HT crops are regulated in an equivalent manner

⁴ According to Canadian definitions, a plant with novel traits is ‘a plant variety/genotype possessing characteristics that demonstrate neither familiarity nor substantial equivalence to those present in a distinct, stable population of a cultivated seed in Canada and that have been intentionally selected, created or introduced into a population of that species through a specific genetic change’.

irrespective of whether they have been produced through modern biotechnology or conventional plant-breeding approaches.

In the United States, GM crops, depending on the trait concerned, are governed either by the Federal Plant Pest Act (enforced by the US Department of Agriculture's Animal and Plant Health Inspection Service (USDA APHIS)) or by the Federal Insecticide, Fungicide and Rodenticide Act (enforced by the EPA). Both of these pieces of legislation require application prior to commercial use and are risk assessment based. Food products derived from GM crops fall under the auspices of the Federal Food, Drug and Cosmetics Act that is administered by the Food and Drug Administration (FDA). In contrast to the situation in Europe, prior consent is not required for the marketing of food containing or consisting of GMOs, although the FDA operates a voluntary consultation procedure whereby producers of GM foods provide information for assessment by FDA scientists prior to marketing. To date, all producers of GM foods have taken part in this voluntary procedure.

Although the legislative approach taken in the United States and Canada is different from that taken in the EU, it is striking to note that science-based ERA is central in all three regimes, and that the information requirements are very similar. Where the regimes differ the most is in the post-market approval period, where only limited conditions apply in North America, in contrast to the stringent post-market monitoring, coexistence, traceability and labelling requirements of the EU.

9.3 Risk assessment of gene flow

As outlined above, the formal risk assessment process requires consideration of two components – hazard and exposure (Poppy, 2004; see also Chapters 7 and 8). These are then combined in order to estimate risk. In some cases, the procedure for combining hazard and exposure is straightforward and quantitative. For example, the risk assessment of chemical hazards is well developed. For a given chemical and use, it is possible to determine the concentration of chemical to which individuals or the environment is exposed, and to use toxicological data to determine the effect of exposure to that concentration. Conversely, given a known toxicity profile, exposure limits, often incorporating an additional safety margin, can be defined. Classically, if risk is considered to be higher than acceptable limits, then either the activity is not permitted, or risk management measures are applied to reduce the level of risk. Risk management can target hazard (e.g. replacing a substance with a less toxic one) or exposure (e.g. reducing the amount of chemical used to limit exposure). While this approach summarises the basic ideas of risk assessment, in practice the process is often more sophisticated. Rather than dealing with simple hazard and exposure measures it is the probabilities of hazard and exposure that are considered, with the resultant risk being calculated as the probability of a hazard being realised.

Risk assessment of GMOs proceeds in a similar manner, although it is often more difficult to impose a strict quantitative framework. However, even though

GMO risk assessments are generally qualitative, hazard and exposure are usually combined in a multiplicative fashion (see also Chapter 10), and ultimately result in an assessment of the probability of potential hazards being realised. This has an important implication central to the risk assessment process. If either hazard or exposure is very low or zero, the overall risk is also deemed to be low or zero. At one level this is intuitive – there is no risk from either a hazard-free substance or from a substance to which one is not exposed – but it is often ignored in discussions of GMOs. In particular, this fact allows uncertainty to be handled. If hazard or exposure is accepted to be low, then uncertainty in the other component has little or no impact on the certainty of the risk assessment. This also provides a focus for the gathering of scientific data to support risk assessments, as the target should be to identify the lowest probability step in the hazard–exposure continuum (Wilkinson *et al.*, 2003b). This step essentially sets the bounds of the risk assessment because even if all the other steps are certain to occur (probability of one), the overall probability of risk is determined by the lowest probability step.

Having defined the approach to risk assessment, from the perspective of gene flow, the key scientific issue for regulators is the extent to which the hazard and exposure components of gene flow are understood.

9.3.1 *How well is the rate of gene transfer known?*

There is a substantial body of information concerning the rate at which major crop species outcross, both with themselves and with sexually compatible wild relatives. In recent years this question has received increasing attention, probably not only because of its intrinsic importance to risk assessment of GMOs but also because studies of the rates of gene flow are, up to a point, generic to particular groups of crops (Wilkinson *et al.*, 2003b). Although one study has reported a differential rate of outcrossing for a transgene and an endogenous gene (Bergelson *et al.*, 1998), this observation has not been repeated. It is widely accepted that the nature of the genes present does not influence the rate of many of the components of gene flow (GM Science Review, 2003). There is also interest in this area from the perspective of coexistence between GM and non-GM crops, as in this case, rates of gene transfer alone are a key issue. A good summary of current knowledge for a range of crops has been produced by the Danish Institute of Agricultural Sciences (DIAS, 2003), and is also contained in various consensus documents produced by the OECD⁵.

Although there is considerable information on outcrossing rates, it is important to recall that this is only one of the components of gene flow as a whole. For gene flow to occur, and to subsequently impact, say, on the population dynamics of a wild relative, more than a successful outcrossing event is required. The product of the outcrossing event must result in the production of a seed, the seed must be viable and the plant that grows from it must itself be both able to compete within in the

⁵ Consensus documents summarise the current state of knowledge of the biology of various crop plant species and are available at http://www.oecd.org/document/51/0,2340,en_2649_34385_1889395_1_1_1_1,00.html.

environment and set viable seed. Any transgenic trait that negatively impacts any of these processes is unlikely to become fixed in a wild population, and even traits that are neutral are likely to disappear in the absence of positive selection pressure (see Chapter 6). Once again this emphasises that, from a regulatory perspective, the key target is to identify the most unlikely event in the exposure chain, and measure its probability to set the upper limit of exposure.

Having said that there is a substantial body of information in this area, much of which has been derived with high scientific rigour, there still remains a difficulty with the data available from a regulatory perspective. For many crops, a key conclusion is the inherent uncertainty in predicting rates of gene transfer. This arises not only from measurement error but also from the stochastic nature of cross-pollination events, particularly between plants some distance apart (see Chapter 3). As discussed elsewhere in this volume, the rate of outcrossing declines with distance according to a leptokurtic function. Not only does this function have a long 'tail', whereby rates of outcrossing do not decline significantly over large distances, there are also rare occurrences of long-distance outcrossing. For example, a study in Australia looking at the transfer of conventionally bred herbicide-resistance traits in oilseed rape found rare cross-pollination events at distances in excess of 3 km (Rieger *et al.*, 2002), while research in the United Kingdom using male sterile bait plants identified a putative cross-pollination event at 26 km, again for oilseed rape (Ramsey *et al.*, 2003). This uncertainty also manifests itself in the consideration of outcrossing between crops and wild relatives. Perhaps the most exhaustive study of this issue to date is the work of Wilkinson *et al.* (2003a) on cross-pollination between oilseed rape and its wild relative *Brassica rapa* in the United Kingdom. Taking into account the spatial overlap of oilseed rape cultivation and the occurrence of wild *B. rapa* populations, the extent of temporal overlap between flowering and empirical measurements and the extent of outcrossing, these authors were able to calculate that annually an average of around 49 000 oilseed rape/*B. rapa* hybrids form in the United Kingdom. While this figure appears to provide a solid base for the risk assessment of gene flow from this crop to its wild relative, there is considerable uncertainty associated with the estimate – the 95% confidence limits (based on 2 standard errors of the mean) are 7 000–91 000 (corresponding to 0.04–0.1% of the total UK *B. rapa* population).

The uncertainty associated with measurement of outcrossing rates does not result from inaccurate or poorly replicated measurements, but is a feature of the phenomenon under consideration. It is perhaps not overly pessimistic to state that uncertainty concerning out crossing rates increases as more studies are carried out. This, coupled with a comparative lack of knowledge of other components of the rate of gene flow, means that in order to carry out a thorough assessment of the potential risks associated with gene flow, it is almost always necessary to consider the potential hazards that might result should gene flow occur. In essence, most risk assessments tend to assume that gene flow occurs and so the focus should be on the consequences of gene flow.

9.3.2 *How well are the consequences of gene flow known?*

Unlike rates of gene flow, which may be measured on a crop-by-crop basis, the consequences of gene flow can only be considered on a case-by-case basis – it is the properties of the particular genes transferred that will lead to consequences, negative or otherwise. Given this, it is perhaps not surprising that knowledge of the consequences of gene flow from GM crops is more limited, and tends to focus on the impact of traits found in currently commercialised GM crops.

It is important to recognise that, at least for gene flow from crops intended for food use, many potential consequences of gene flow into non-GM crops can be discounted. If the GM crop itself has been assessed as safe for use in human food or animal feed, then it is unlikely that gene flow will result in the presence of components in a crop that would be considered harmful. Of course there are exceptions to this rule that would need to be carefully considered at the risk assessment stage, and there are particular issues associated with crops modified for the production of material that is not intended for food or feed use. Having said this, there are many precedents for the production of industrial material in specific varieties of crops produced through non-GM breeding (e.g. high erucic acid oilseed rape).

The primary source of potential hazard resulting from gene flow from GM crops is that this leads to adverse effects on populations of wild relatives of those crops, or negative consequences on organisms that interact with those wild relatives. If, for example, a transferred gene were to lead to a dramatic enhancement in the fitness of a recipient wild relative, then this might lead to a change in the population biology of the species concerned, which might itself impact on community structure. As a worst-case scenario this could lead to the extinction of particular species or to a reduction in the genetic diversity of species, with the genotype that received a beneficial transgene dominating at the expense of others. The task of risk assessment is to establish the likelihood of such a chain of events occurring (Wilkinson *et al.*, 2003b). As argued previously, the probability of such a sequence will be limited by the event in the sequence with the lowest probability, and much attention has been focused on the likelihood of specific transgenes to cause a significant change (either enhancement or reduction) in the fitness of recipients receiving the transgenes.

From a regulatory perspective, traits that reduce fitness in wild relatives can be considered to constitute low environmental risk. Individuals carrying these transgenes will be less fit than their siblings that do not contain the transgene (by definition) and so will therefore contribute proportionately fewer progeny into the next generation. Transgenes of this type will not get fixed in the population except on rare occasions by genetic drift, and, assuming that those individuals that receive the transgene is not determined genetically, the genetic structure of the population will not be altered (see Chapter 6). It is also the case that for all the presently considered GM crops the rate of transfer of genes to wild relatives is low, and so the impact on the population of the recipient species is likely to be negligible. An example of a trait that is expected to behave in this way is male sterility.

In comparison with transgenes conferring reduced fitness, those that lead to an enhancement of fitness give more cause for concern, so that an assessment of the extent to which transgenes could enhance the fitness of wild populations is a reasonable and pertinent question to address. There is, however, comparatively little information to aid the regulator in this context. Not only is the information concerning the effect of specific transgenes limited, but there is also limited theoretical knowledge concerning the factors that determine the fitness of plants in the natural environment. Some attention has been focused on the factors that determine weediness in plants, with the production of checklists of factors that might contribute to this complex trait. However, the factors contained on these lists tend to be rather self-evident (e.g. high rates of seed production and apomixis), and are often complex traits that are difficult, if not impossible, to link to specific transgenes. This approach also suffers from being entirely qualitative in the sense that the relative importance of particular traits to the development of weediness is not known. Key questions for regulators are as follows:

- For particular species that may be the recipients of transgenes from GM crops, what are the key genetic determinants of fitness and geographical range?
- To what extent is fitness determined by one-sided limitations that could be alleviated by the introductions of one or a few genes?

Our ability to answer these questions will limit the extent to which the impact of novel genotypes in agriculture, including the products of both conventional and GM plant breeding, can be thoroughly assessed.

Although there is a general area of uncertainty regarding the impact of transgenes on wild populations, it is possible to make sound regulatory decisions for the existing generation of GM crops. In some cases, this is because of knowledge of the exposure component of the risk equation. For example, maize is not considered to have any wild relatives with which to outcross in the European flora (OECD, 2003), so that the issue of impact on wild populations through gene flow can be discounted. In other cases, the nature of the trait means that impact on recipient populations is negligible. For example, herbicide-tolerance traits are thought unlikely to impact in the absence of the herbicide. However, it is important to consider the potential impact of new traits or the transfer of currently used traits into new crops. The use of insect resistance traits in sunflower in North America has received some attention – it has been shown that the presence of insect resistance traits in wild sunflowers leads to an enhanced production of seed, implying that insect damage limits fecundity in the natural environment (Snow *et al.*, 2003). While it does not necessarily follow that increased fecundity equates to increased fitness, this system is clearly of interest and merits further work (see Chapters 6 and 8).

The consideration of the impact of gene flow on wild populations presents an interesting dilemma for the regulator. The only robust way of assessing the impact of transgenes in wild species in the environment is to allow the direct testing of fitness under realistic environmental conditions, but this may be considered itself to be a risk to the environment (see Chapter 6). Clearly, issues of scale are important here, and

small-scale studies may be carried out without risking significant impact provided effective isolation of the test population(s) is possible. But it is also necessary to build sufficient theoretical knowledge of the population biology of plants, so that ecological fitness can be estimated prior to releases.

9.4 Conclusions

Consideration of the rates and consequences of gene flow plays an important part in the regulation of GM crops, and it is necessary that regulators have access to accurate scientific information in this area. As the range of GM crops and the traits that are transferred to them increase, further research will be needed, at least while international regulatory regimes remain in their current form.

In many contexts, another role of regulators will be to design, implement and enforce measures that limit gene flow, either for the purposes of risk management, or, more commonly, to allow GM and non-GM supply chains to coexist thus ensuring consumer choice. While there is currently good experience with agronomic measures to limit gene flow, such as separation distances, or barrier crops, it has also been suggested that technological solutions may also be possible. These solutions generally require further traits to be transferred into the GM plants, and examples include plastid transformation and the engineering of male sterility or altered seed viability (see, for example, Scherthamer *et al.*, 2003). While many of these approaches bring the real possibility of complete genetic isolation of GM crops (especially if multiple approaches are taken simultaneously), it is important to consider that these novel traits may themselves raise risk assessment issues. Unless genetic isolation is required for risk management purposes, it is generally regarded as best practice to minimise the amount of foreign DNA present in GM crops (ACRE, 2001).

It is likely that gene flow will remain an important issue for the regulation of GM crops for some time to come. However, what is less clear is the extent to which the concerns expressed around GM crops become extended to their conventionally bred counterparts. One consequence of the attitude of the European consumer to GM crops is that there are active breeding programmes aimed at producing traits like herbicide tolerance without the use of GM technology. Will these novel crops be subjected to the same scrutiny as their GM cousins? Or will we continue to regulate on the basis of the process whereby crops are produced not on their actual environmental impacts?

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10 Risk assessment of GM crops – does the road ahead need to be long and winding?

Guy M. Poppy and Michael J. Wilkinson

10.1 Setting the scene

There is nothing new about gene flow from agricultural crops to their wild relatives, but it has received increased attention since the advent of GM crops. Throughout this book, similarities and differences relating to measuring and characterising the nature of the risks associated with gene flow from conventional as opposed to GM crops have been explored. It is also important to realise that gene flow is just one of the environmental factors leading to risks associated with GM crops, and as such, gene flow needs to be considered in conjunction with issues relating to biodiversity, non-target effects and extensification versus intensification of agriculture (see Chapters 6, 7 and 8 and Poppy, 2000, 2004).

In order to understand why there is so much concern about gene flow and other risks deriving from the commercialisation of GM crops, especially within the European Union (EU), it is perhaps important to raise broader issues relating to agriculture and the environment. Perceived or real differences about the role of agriculture in society, especially in relation to the wider issues of land management, may partially explain why gene flow from GM crops is receiving such intense attention within EU countries compared with North America. We will address some of these wider issues in this concluding chapter, as well as attempt to offer new approaches to the risk assessment of GM crops.

10.2 Managing the land for multiple users

The world population currently stands at 6 billion people and it is estimated to rise to between 7 and 12 billion by 2050 (UN Population Information Network, 2002). Although the precise number depends on the success of programmes established to curb population growth, there is no doubt that the number of people in the world will grow substantially over the coming decades. While there is heated debate about our current ability to feed the world and whether increased production or better distribution in isolation from each other will help, there is little disagreement that we will need to increase production if we are to attempt to provide enough food in the future.

It is impossible to cover this topic in detail in this chapter but Table 10.1 illustrates some fundamentally important concepts relating to land availability and productivity. The take-home message is that agriculture needs to be relatively

Table 10.1 Number of people that could be supported by food produced by different agricultural methods

Production system	People supported per ha
1.5 billion hectares of arable land	Needs to support 4 per hectare
Wheat Canada	6
Wheat UK	21
Shifting cultivation, PNG 1962–1963 (low input)	0.3
European open-field system, England 1920–1940 (low input)	1
Southern India, 1955 (low input)	2
3.4 billion hectares of grazing land	Needs to support 2 per hectare
Beef cattle, lowland England	1
Migratory pastoralists, Kenya 1981–1982 (animals)	0.005

Note: High input farming is given in bold and the remaining represent low input farming; modified from Newman, 2000.

intensive to feed the current population, let alone that projected for 2050. While there is a role for low input farming, it is hard to see how it can feed a world, especially one consuming so much meat.

The green revolution radically changed agriculture and undoubtedly saved many lives in the short term (Borlaug, 1970). Although there is much cynicism about the long-term benefits of the green revolution, and GM is being dubbed as a second green revolution, it must not be forgotten that it would require 40% more land to produce the same amount of food produced in 1990, if we used pre-1960s agricultural techniques. Coupled with this need is a strong desire for food production to be sustainable in the long term. Given the sheer scale of these challenges, it seems naïve to propose that adequate progress can be achieved without the use of all forms of agricultural high technology, including biotechnology.

It is also of interest to consider the adoption of GM crops in the United States compared with that in the European Union. Although regulatory systems differ (see Chapter 9), there also seems to be a marked difference between the peoples and systems of the two in terms of risk perception and risk aversion. While one may propose that the US public are generally more accepting of multinational corporations and encourage risk-taking if the benefits are large, one should also consider the different approaches to agriculture and the environment. Figure 10.1 illustrates differences in land use between the United States and Europe. It is very noticeable that agriculture and the countryside supporting biodiversity, as perceived by the public, overlap more in Europe (Hails, 2002; Poppy, in press). Coupled with recent farming catastrophes in Europe, in particular the United Kingdom, the European public are more wary of the continuing intensification of agriculture and how the landscape, which is the countryside they utilise, may be affected (Eurobarometer, 1993). A real challenge facing the world is how to produce enough food from the land while maintaining the valued aspects of the environment in which we live. This issue has been prominent in Earth Summits from Rio through to Johannesburg.

Although it is important to highlight differences between the continents' acceptance of GM technology, it is important that we look forward and try to see where

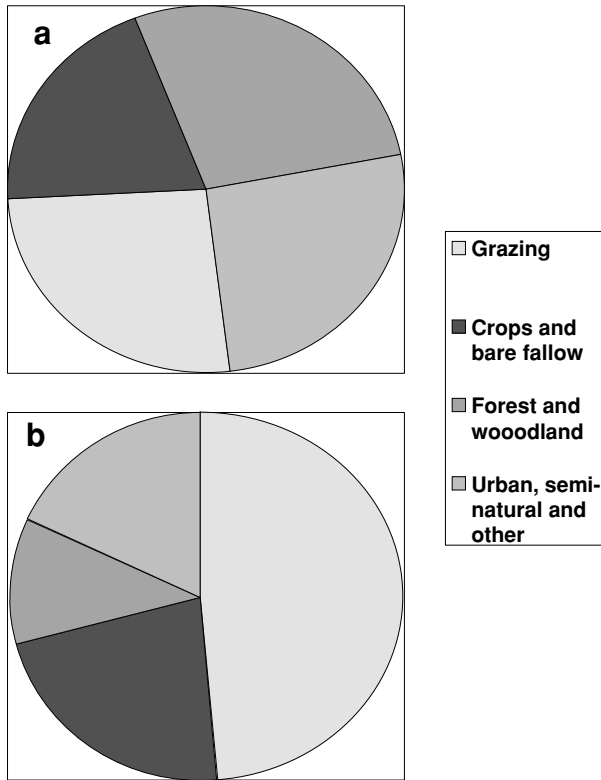


Figure 10.1 Land use in the (a) United States in 1997 and (b) the United Kingdom in 2000. (Modified from Hails, 2002.)

harmonisation and efficient risk assessment frameworks can be developed. In this final chapter of the book, we outline issues relating to risk assessment and suggest approaches and opportunities that may allow us to progress forward in a precautionary fashion, rather than remain stationary for precautionary reasons (for details of precautionary principle see Gray & Bewers, 1996, and Prime Minister's Strategy Unit, 2003).

10.3 Global strategies for risk assessment

Divergence in the regulatory processes adopted by Europe, the United States and other stakeholder nations in relation to the commercial release of GM crops has huge importance for international trade and has the potential to threaten the future economic viability of biotechnology in agriculture (Arntzen *et al.*, 2003). To a large extent, the hope for global harmonisation in relation to the regulation of GM crops is perhaps a naïve one, especially given the wide variety of legal structures between

countries and the divergent environmental priorities. At the same time, however, biological organisms do not respect political boundaries and so decisions made in one country have the capacity to eventually impact on others in the region or even globally. Thus, there is a common need at this point to make progress towards defining more clearly what we wish to achieve from legislation.

The pattern of land use varies widely between countries and can be crudely divided into agricultural, urban and conservation–recreational areas (see Figure 10.1). What we want from our land depends largely on where it is and on what our priorities are for the area concerned. For instance, in urban areas, the creation of jobs, homes or of improved infrastructure may be the highest priority, whereas in an area dominated by intensive agriculture, changes leading to increased profitability of farming may be important. Priorities in conservation areas probably lie in maintenance of the existing ecosystem (notwithstanding natural changes in community structure, including some local extinctions), often in the context of recreation. History and topography of the landscape means that in many parts of the globe, including the United States, there is often a relatively sharp distinction between the predominant usages in different regions, and so the process of setting local priorities is comparatively easy. This is in stark contrast to the situation in Europe, where the landscape management is highly integrative, with conservation, recreation, farming and to some extent even urban development frequently coexisting in the same area (see Figure 10.1 and Hails, 2002). In such circumstances, measures taken to enhance one function can have negative implications for other land use functions. For instance, expansion of urban development reduces land available for conservation or food production. Indeed, McKee *et al.* (2003) examined the relationship between human population density and the number of threatened mammal and bird species by nation. Multiple regression analysis revealed that two variables, human population density and species richness (of birds and mammals), account for 88% of the variability in log-transformed densities of threatened species across 114 continental nations. They went on to predict that the mean number of threatened species per nation would increase 7% by 2020 and 14% by 2050. While some of this correlation may be attributed to increased assignment of land to agricultural production, other land uses (urban and recreational) will also be responsible. It follows from this that pressure on land for conservation will inevitably increase unless agricultural land becomes even more productive. Likewise, measures designed to increase food productivity may impact negatively on biodiversity in the area, or conversely, procedures aimed at encouraging conservation of biodiversity (e.g. reduced herbicide application) may result in a drop in food productivity. This means that land management decisions made to encourage one aspect of the landscape should also take cognisance of the impact on other land uses. This then necessitates the setting of local priorities that may be different from other regions or nations, and may itself be subject to cultural, political or economic change. The importance of deliberate policy decisions in shaping the landscape is also likely to differ profoundly between nations and regions, with some nation states adopting a ‘hand-off’ approach, while others employ an active interventionist strategy. For instance, one may expect some

contrast here between the United States, in which state priorities will probably be set nationally, and the European Union, where individual member states seem likely to exert more control over local priorities (see Chapter 9). In any event, change to the landscape is an inevitable consequence of human development, natural evolutionary processes, climatic change and economic forces. This means that the absence of decision making does not equate to conserving the status quo and also that legislators should have a clear idea of where their priorities lie when creating new legislations and an understanding of the likely implications for non-target applications. It is in this context that we must consider the future needs for the regulation of GM crops. In particular, full cognisance must be taken of the broader implications to all land use applications within the context of a given set of priorities of a decision to accede a submission and equally of a decision to refuse permission for commercial release. This includes accepting responsibility for possible perturbations in neighbouring regions where priorities may differ.

10.4 Relative importance of environmental changes arising from GM crops

The possible consequences arising from the cultivation of GM crops must be weighed against existing baseline variation (e.g. that caused by genetic variation between existing cultivars or by differences in farm practice) and also against the scale of change imposed by other sorts of alterations to the landscape. When viewed in this context, it is probably insufficient simply to demonstrate a negative effect caused by the cultivation of a particular GM cultivar when compared with its non-GM equivalent, particularly if this difference is negligible when set against the variation that exists between existing non-GM cultivars. This point becomes especially germane in instances where the GM cultivar offers significant environmental benefits in other areas of importance. On the other hand, it is undoubtedly also important that more thought be given to the possible longer term implications arising from the widespread release of a transgene into the agrio-environment. The nature and significance of possible environmental perturbations arising from the cultivation of GM crops vary widely, and are heavily dependent upon the crop, geographical location and action of the transgene(s) product (see Chapters 5, 6 and 8). There is a wide range of possible environmental concerns relating to GM crops and include, among others, those listed in Table 10.2.

The importance that is attached to these outcomes is to some extent reliant on the local priorities for land use. For instance, a region or country in which agricultural output is deemed to be of greatest importance, concern and, in all likelihood, associated risk assessment research and legislation, will tend to focus on the possibility of an aggravated weed, pest or disease problem. If, on the other hand, conservation of the natural ecosystem ranks highest in terms of priority, then one would expect more effort directed towards anticipating changes to on-farm or wild plant and animal communities. Thus, different regions/nations need to set formal goals,

Table 10.2 Some key areas of concern arising from gene flow from GM crops

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1. GM cultivars will require detrimental changes to farm practice
 2. GM crops will become pernicious volunteer weeds
 3. Gene flow will compromise the economic value of non-GM cultivars
 4. GM HT crops will accelerate the evolution of herbicide-tolerant (HT) weeds
 5. Drought-tolerant or salt-tolerant GM cultivars will encourage encroachment onto ecologically sensitive land
 6. Gene flow to weedy relatives will exacerbate existing weed problems
 7. Control of GM weedy relatives may detrimentally impact the ecological balance to the on-farm community
 8. Insect-resistant or disease-resistant GM cultivars will encourage the evolution of new, pernicious strains
 9. Pest-resistant GM cultivars will cause displacement of pests onto alternative wild hosts and cause unwanted changes to the community
 10. Gene flow to wild relatives may disrupt the ecology of the recipient community and lead to the decline or extinction of one or more species
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reflecting their environmental priorities as the central part of their ERAs (see below and Chapter 7). One of the key problems with all systems of regulation currently in force around the globe lies in the total absence of explicitly ranked lists of priorities with regard to land use. The lack of a specified ideotype for the landscape (i.e. a view on precisely what it is that we least want to change in the landscape, and what we would like to change) means that there is an innate danger not only that are specific risks relating to particular GM cultivars assessed but that the relative importance attached to each expected change is also likely to vary. Hypothetically, this consideration would not be an issue if it were possible to commission research to comprehensively address all scenarios of risk across all possible submissions. However, the list of possible concerns listed above is certainly incomplete, and each of these should be further subdivided into many more precisely defined hazards (e.g. gene flow from GM Bt (Cry3a) oilseed rape to wild *Brassica rapa* leading to the significant decline of a named beetle from the United Kingdom) (see Chapters 6, 7 and 8). This means that there would be a strong need for high-quality data that has relevance for the decision-making process relating to a large number of possible hazards across all submissions of every crop and in all geographic regions. In the medium term, this task is made all the more implausible by the rapid growth in the diversity of novel traits conferred by constructs and by the tendency towards the creation of GM cultivars with multiple inserts (e.g. see James, 2003). At the same time, the number of GM crop species becoming available is increasing, as is the number of countries that permit the cultivation of GM cultivars on a commercial basis. If GM risk assessment research is to maintain a genuine role in aiding the regulatory process (as opposed to a perfunctory provider of excuses that justify decisions made on the basis of political expediency), it follows that there is an urgent requirement to make radical decisions about where the priorities lie. Ideally, these should be made in relation to assessment endpoints (targeted hazards) of highest importance within the framework of regional ERAs (see below, Chapter 7 and Poppy *et al.*, in

press). The first task then is to identify those environmental changes that are truly unacceptable so that research effort can be appropriately prioritised. On this point, it must be remembered that the more changes that are included in the highest ranking category for a particular area, the slower the progress will be and the lesser will be the ability to predict likely outcomes. One option might therefore be to opt to delay approval for release almost indefinitely until comprehensive data sets are available for all plausible assessment endpoints evaluated. However, such an action is itself not without the potential for seriously negative consequences. On one level, protracted delay may impact on the global economic competitiveness of farming within the legislative region, depending on the crop, global prices for the marketable product and the financial benefits accruing from the use of the particular GM line. There is equally scope for negative environmental impacts through the decision to defer uptake of a benign GM cultivar. For instance, in regions where food production is at or below subsistence levels and output is limited by pests, diseases or post-harvest losses, the shortfall can be met only through expansion of productive land (generally at the expense of natural habitats) or through the potentially damaging use of more intensive application of prophylactic chemicals and intensive farm practices. The decision is clearly less stark in wealthy temperate regions, where the prospect of reduced agricultural production is generally of less importance to the overall economy and locally produced food is often supplemented by imports. Furthermore, market forces in these regions frequently provide a small premium for organic produce, and consumer resistance to GM technology, particularly in Europe, means that the market for products containing GM produce can be restricted. What is less clear is whether these trends are likely to persist, and if so what is the long-term effect that they will have on farming patterns? Clearly, in poorer regions where food production is limiting, the amount of land that is used for food production will tend to expand or contract according to a combination of demand and productivity. In crude terms, the area of cultivation will grow as the population expands unless increases in productivity per unit area can compensate. The relationship is less direct in the wealthier nations, since any shortfall can be accommodated for by increased imports. (For figures on UK/US and other countries' food self-sufficiency, see FAO, 2004.) This potentially allows for less productive systems such as organic farming to become increasingly prevalent. Paradoxically, however, while on-farm biodiversity in wealthy temperate regions may benefit from such a change, the need for increased imports may well mean that conservation land elsewhere is converted to agricultural production. The ecological significance of such displacement could be extremely serious if, as present, much of the imported goods originate from species-rich tropical regions. Thus, there is something of a conundrum. On the one hand, the abandonment or severe restriction of GM technology, at least by some regions, may seem to offer a precautionary stance that protects local on-farm biodiversity but may threaten the long-term security of more bio-rich communities elsewhere in the world by food production displacement (Borlaug, 1997; Poppy, *in press*). On the other hand, as GM technology continues to expand so that crop-construct diversity increases continually, the current case-by-case consideration of all possible negative changes

to the environment becomes superficial, with potentially serious and irreversible environmental consequences. The most logical way forward is to prioritise those aspects of the landscape that we most wish to protect from detrimental change and focus research effort on assessing risks relating to these environmental key-point hazards. It is important, however, that a holistic approach is adopted in anticipating such changes. In the longer term, there seems little point in investing time and resources in determining whether cultivation of a particular GM cultivar will lead to extinction of an organism deemed to be important, only to find that extinction occurs as a direct result of some other land management decision. It is therefore desirable to predict all categories of land management changes that could lead to extinction or severe decline of organisms or communities identified as important on the grounds of ecological importance, evolutionary isolation or even social significance. This includes predicting the impact of, among others, changes on farm management such as food subsidy alterations or introduction and/or withdrawal of herbicides and pesticides; construction projects; importation of alien plant and animal species introduced for biocontrol or recreational purposes; the introduction of a new crop; and the change in land use (e.g. conversion from food production to forestry). Given the large number of drivers of change and the potentially large numbers of organisms/communities that require attention, there is clearly need for radical rationalisation and targeting of effort. At the simplest level, the long-term monitoring of target organisms/communities for significant changes in population size or in community structure offers a contemporary and a posteriori approach that can nevertheless have value for assessing the effects of reversible changes to land use such as those likely to cause altered farm management. However, such a strategy has limited utility for irreversible changes such as gene flow from GM crops to wild relatives. In these cases, it is important to assimilate a predictive understanding of the dynamics of the organism or community concerned so that the significance of perturbations can be anticipated. To achieve this, we need to accumulate generic data sets that can be applied to as many scenarios as possible (Wilkinson *et al.*, 2003). In the remaining part of this chapter, we aim to illustrate how this could be achieved for the assessment of risks associated with gene flow from GM crops.

10.5 History of GM risk assessment

Concerns over possible negative environmental impacts arising from the cultivation of GM crops have ranged in scope from small-scale laboratory experiments that aim primarily to identify possible hazards (hazard identification studies) through to large-scale, multidisciplinary efforts that seek to assess the likelihood that a specified hazard will occur (exposure and risk assessment studies) (see Chapters 7 and 8). What has been generally lacking is co-ordination between research efforts, and the emphasis has been on science that relates to risk assessment rather than on the acquisition of information for the purpose of decision making (see Chapter 7). In consequence, there has been a rather eclectic mix of studies addressing issues with

varying relevance to the risk assessment process. There have been two key drivers for this research and these have differing limitations with respect to meeting future needs of GM risk assessment.

10.6 Hazard-led research

This category of risk assessment research is generally initiated by the discovery of a potential hazard. Perhaps the most celebrated example were the risk assessment studies triggered by the study of Losey *et al.* (1999). These authors used a simple laboratory feeding experiment to indicate that the presence of pollen from GM maize could negatively influence survival of monarch butterfly larvae when dusted on their normal food plant. They went on to infer that the presence of GM Bt maize could therefore constitute a threat to the long-term survival of the monarch in North America. While this work was subject to criticism (Gatehouse *et al.*, 2002), the identification of this potential hazard, coupled with the intense media interest that followed, led to an excellent series of exposure papers that evaluated the likelihood that the hazard would occur (Sears *et al.*, 2001, and other papers in special edition of *PNAS*). The work culminated in a realistic estimation of the risks associated with the cultivation of GM Bt maize on monarch butterfly numbers (Sears *et al.*, 2001). The key weakness in routinely adopting such a responsive approach to risk assessment rests in its reliance on the imagination and quality of the initial hazard identification study. Inevitably, priority will be given to those hazards that happen to be identified first or else involve the most emotive consequences (regardless of the likelihood of occurrence or ecological importance). The weight of evidence required to identify a possible hazard is an order of magnitude less than is needed to perform a full-scale evaluation of exposure. It is therefore vital that effort is made to prioritise exposure research. The currently favoured tactic is to apply a tiered strategy (Poppy, 2003; Dutton *et al.*, 2003; also see Chapters 7 and 8) to evaluate exposure once such hazards have been identified. Here, initial laboratory experiments mimic worst-case scenario conditions and further, progressively more realistic experiments are applied only to those hazards that are substantiated. Thus, expensive full risk assessments are only implemented to hazards that are deemed possible even under realistic conditions. Nevertheless, while the adoption of a tiered strategy to evaluate exposure reduces the misapplication of resources to insignificant hazards, the danger remains that time and effort unnecessarily spent on an unimportant hazard detracts from hazards of highest priority.

10.7 Regulation-led research

There is a real and pressing need for regulators to obtain data relating to GM lines currently under consideration for release. Here, less importance is placed on the underlying principles influencing the nature and extent of change, and more emphasis

is given to empirical observations on whether the cultivation of the specific GM lines induce greater or less change than do conventional cultivars of the same crop. The landmark Farm Scale Evaluations study in the United Kingdom adopted this broad strategy to test whether GM HT crops of sugar beet (*Beta vulgaris*), maize (*Zea mays*) and both spring- and autumn-sown oilseed rape (*Brassica napus*) effect on-farm biodiversity compared with a conventional cropping system. A split-field design was used in which GM and non-GM equivalents were grown in adjacent halves of the same field, with the number of such sites for each crop replicated at 60–75 locations across the United Kingdom. This design allowed direct comparisons of the abundance and species diversity of the in-field weed flora and fauna (Firbank *et al.*, 2003). The work found that the GM HT maize plots consistently contained a higher on-farm biodiversity than the non-GM equivalent, whereas in broad terms the reverse was true for sugar beet and oilseed rape (series of papers in special edition of *Philosophical Transactions of the Royal Society* (Royal Society, 2003)). These data clearly had direct relevance for the priorities of the UK regulators and undoubtedly played a key role in the decision to provisionally approve release of the GM HT maize but to defer decision for the other crops. This approach is a statistically powerful means of examining effects on on-farm biodiversity but is extremely costly, and has limited predictive utility for other GM lines of the same crop, even for GM lines containing different forms of herbicide tolerance. Furthermore, in the case of the approved GM HT maize, there was no need to address the issue of gene flow to wild relatives since these are absent from the United Kingdom. In coming years, expansion in the diversity and complexity of constructs introduced into GM crops may also render such intensive and expensive studies an impractical proposition. In any case, it is difficult to apply this kind of study to examine potential hazards associated with gene flow to wild relatives.

10.8 The way forward

There is another broader issue here that is illustrated by terms of reference in which the FSE study operated: Is species biodiversity *per se* the only appropriate measure on which to judge unwanted ecological change (see Poppy, in press)? The problem with species biodiversity lies in the fact that, by definition, all organisms are considered of equal importance. The basic ethos is epitomised by the phrase ‘the more, the merrier’. While this may be an appropriate measure in bio-rich tropical environments, its usefulness is more questionable in a temperate setting. Taken in the strictest sense, some of the most biodiverse temperate habitats are wastelands such as docks and building sites, since these often contain an abundance of ruderals, hybrids and alien species. Conversely, many of our most treasured habitats, including wetlands, fixed dunes and bogs, are comparatively species-depauperate. Indeed, in these communities, an increase in species diversity is likely to be symptomatic of a possible breakdown in community structure and/or function, and so should be

viewed as undesirable. Biodiversity can also be measured in terms of genetic biodiversity within a species and ecosystem diversity. These types of biodiversity need to be measured at different scales and may vary in importance according to the question(s) being asked. For example, if the species biodiversity on a farm was increased at the sacrifice of turning more forest into farmland, then species biodiversity would drop at a scale that included the former forest as would overall ecosystem biodiversity (see Poppy, in press). This raises the question as to what is best for biodiversity, in its broadest sense – small pockets of intensively farmed land with large areas of unfarmed land, or extensive areas of low-intensity agriculture resulting in less land for other needs? This is the type of question we should be asking, but the answer may be difficult to achieve.

At best then, biodiversity provides a crude measure of environmental change but no indication as to whether that change is desirable. At worst, its use as a target may precipitate action that in reality is detrimental to a community or to the survival of a particularly valuable organism. Herein lies the problem. It is impossible for us to assess risks of unwanted change unless we are able to specify what it is that we do want. It is therefore vital that we start to be explicit about what aspects of the environment are valuable to us and which aspects are less so. In this way, we should be able to set measurable goals against which risk assessment for GM crops or indeed any regulated aspect of land use can be evaluated. In Chapter 7, Raybould and Wilkinson describe the concept of endpoint assessment (also see Poppy *et al.*, in press); broadly speaking, these are goals that are set to protect a specific entity from excessive detrimental change. For instance, an assessment endpoint may set a minimum acceptable population size for a protected species in a defined area. As mentioned above, careful thought must be given to the systems developed to designate and rank assessment endpoints, so that effort is focused primarily on targets that are locally important. This will clearly involve value judgements and will take into account local and national priorities. It is also possible that the ranking and even recognition of assessment endpoints may change with time, either because of changed priorities or else because of a change in the entity (e.g. if an endangered species becomes abundant or extinct). Consideration of all assessment endpoints allows for an environmental risk assessment (ERA, also known as ecological risk assessments) to be performed, such that the decisions made relating to the environment cause least disruption to the assessment endpoints deemed to be important. This ethos allows for progression from the potential paralysis induced by the precautionary principle, in which there is no weighting of ecological or environmental change, and moves towards a more proactive approach for conservation of the landscape in which we live (see Poppy, 2005). With regards to risk assessment of gene flow from GM crops, Raybould and Wilkinson (Chapter 7) point out that for ecological conservation, emphasis should be placed on specifying endpoints relating to toxicity of the transgene product, to the consequences of invasion (of the crop or wild relative) and to interactions of the transgenic recipient of gene flow with other plants and animals.

To do this, we need to examine both the exposure and hazard elements of the risk equation (see Chapters 7 and 8 and Poppy, 2004). The definition of hazards should relate directly to assessment endpoint priorities set in the ERA. Such ranking of hazards reflects national and local priorities and so ensures that risk assessment effort is targeted to the aspects of the environment deemed to be most valuable. Needless to say, this will vary between regions and nation states but by definition means that exposure will only be assessed for priority hazards.

For exposure, we should take full cognisance of the reproductive biology of the crop and wild relatives (see Chapters 3 and 4) before adopting a progressive approach in which we assemble increasingly predictive assessments of the extent, pattern and distribution of introgressive hybridisation, culminating with models predicting the exposure of a recipient at the national scale. When assembling such data, it is important to produce data in a form that aids the relevant regulatory authorities in the decision-making process (see Chapter 9). In most instances, it will be clear at an early stage whether gene flow is likely to occur at significant levels. If early experiments suggest no or negligible gene flow (say, on the basis of cross-incompatibility or non-synchronous flowering), then risk can be deemed low on the basis of minimal exposure. On the other hand, if early experiments suggest otherwise, regulators and risk assessment scientists should adopt a working assumption of widespread gene flow, and simultaneously progress to studies examining the influence of the transgene on the fitness of the hybrid and introgressed individuals of the recipient species (see Chapters 6 and 8). Here, the goal is to predict the effect of the transgene on the life history and population dynamics of the transgenic recipient plant (see Chapter 6) and on the interactions between the recipient and other organisms (see Chapters 7 and 8). Quantifying the former will help determine whether the recipient is likely to increase in abundance within its native habitat and so impact on cohabitant species, or else expand its ecological range to invade new communities (see Chapter 7). When assessing the chain of events (exposure pathways; see Chapter 8) leading to the specified hazards (assessment endpoints), it is vital that a progressive, tiered approach is adopted (Chapters 7 and 8), in which initial experiments define worst-case scenarios and in subsequent experimental tiers, conditions become increasingly realistic. In this way, as soon as it becomes evident that there is a negligible probability of hazard realisation (i.e. exposure approximates to zero), exposure assessments should terminate and efforts switched to address other endpoints.

To conclude then, we argue that the continued development of GM technology will dramatically increase the demand and complexity of the GM risk assessment process. In order to avoid paralysis, it will be necessary to adopt a stratified and clear policy for systematic data gathering to aid the decision-making process. To this end, we advocate ERAs in which priority assessment endpoints are specified and used to define hazards that warrant an assessment of exposure. That way, we engage an active role in the management of our environment rather than resorting to a cataleptic avoidance of any decision that might lead to anonymous but detectable changes to the ecosystem.

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