SESSION 4
TRANSGENIC ORGANISMS

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The current status of agronomic traits

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ABSTRACT

Agronomic traits have made a significant contribution to consumer needs via the provision of a safe, secure, and affordable food supply. The continued development of agronomic traits that enhance yield and protect production from the ravages of pests, diseases, and environmental stress is critical. However, it is unlikely that conventional genetic manipulation via breeding and recurrent phenotypic selection will be sufficient to meet future demands. New biotechnological techniques offer unprecedented precision, speed, control, and order-of-magnitude improvements in the germplasm base. Commercial transgenic crops are already successful from an agronomic perspective, and are being grown on about 40 million hectares. Simultaneously, these "trait enhanced crops" have unleashed a complex socio-political reaction that threatens the future of scientific progress and global food security. In the development pipeline and being extensively evaluated in field trials, are several traits that will impact yield potential, and harvestable yield. At the research stage, there is evidence that production efficiency may be improved by using crop biotechnology tools. Efficiency improvement, where fewer input resources would be used for every unit of required output, will provide environmental advantages that may help mitigate other anthropogenic impacts and allow more sustainable practices.

INTRODUCTION

Each year, seed companies introduce hundreds of new varieties and hybrids, contributing to the continuous improvement in crop production. For the major crops, any single annual incremental improvement is typically small and variable. Nevertheless, the overall average yield shows a linear increase with time when viewed over several years (McLaren, 2000a). Historically, this improvement has arisen largely due to genetic manipulation via complex breeding schemes that include several generations of recurrent selection from the gene pool. Selection has been at the level of the phenotype using either visible criteria, or quantified attributes such as statistical improvements in measured yield of harvestable components. The results have been enhanced features that can be classified as "agronomic" traits.

To some extent, improvements in compositional ("quality" or "output") traits have also occurred. However, success with these traits has been more difficult to achieve: due to limitations in conventional genetic manipulation and in inadequate abilities to measure composition at a high-throughput level. The material reviewed in this paper will focus on agronomic traits and explore the various impacts that newer transgenic techniques are creating, compared to traditional genetic manipulation methods.

Agronomic Traits

Agronomic traits are defined here as features of a crop plant that include one, or more, of the following:

1. An improvement in yield potential.
   e.g. light interception, net carbon fixation, lower stress impact.

2. An increase in harvested yield.
   e.g. protection against losses due to pests and diseases, or larger harvest index.

3. Result in a higher harvested yield per unit of input resource used.
   e.g. efficiency of water or land use, or less nitrogen required per unit production.

In some conventional cases, improvement has been via the introgression of disease resistance genes and a good correlation can be observed between the inserted genes, the intermediate effect (fewer pathogen lesions), and the ultimate effect (harvested yield). However, in many other cases, the link between genes and the phenotype is not so evident – there has been a "black box" between genetic manipulation and the desired outcome. Success has been achieved one small step at a time, and mainly because of trial-and-error experiments combined with massive field selection programs.

The science of biotechnology has provided tools and techniques that can be used in plant breeding to insert a known genetic sequence. Since the first transgenic plants in the early 1980s (Horsch *et al.*, 1982), the techniques have continued to develop and can now be used for very precise alterations to endogenous DNA, insertion of one or several stacked genes at known locations, tight regulation of gene expression, and detailed study of genetic effects through isogenic comparisons and/or controlled knock-out experiments.

The first transgenic crops in commercial production have been remarkable in a number of respects. First, they have been an outstanding technical success, second, they have been a great commercial success and, third, they have become the platform for an unprecedented debate over crop production, food supply, scientific progress, regulatory processes, and a host of other socio-political causes. The term "GM" (genetically modified) continues to be used by many people when referring to transgenic crops, and the GM concept has recently taken on negative connotations in some countries. Of course, all crops are Genetically Modified: some via chemical mutation breeding, and certainly via random gene recombination and recurrent phenotypic selection. It would be much better to use a realistic and descriptive distinction for transgenic crops, such as TRAIT ENHANCED CROPS (TEC). This terminology allows for the development of meaningful acronyms for differing situations, for example:

S-TEC = specific Trait Enhanced Crop
IdP-TEC = identity preserved TEC
A-TEC = agronomic TEC
DP-TEC = disease protected TEC
VA-TEC = value added TEC
HT- TEC = herbicide tolerant TEC
IP-TEC = insect-protected TEC
ST-TEC = stress tolerant TEC

COMMERCIAL AGRONOMIC TRAIT ENHANCED CROPS

In 1999, commercial transgenic crops were all A-TECs and more specifically were all crop protection traits. Seven different A-TECs were grown, 12 countries had officially approved commercial areas, and the global total area was almost 40 million hectares (James, 1999). In the US, the area of A-TECs has expanded to almost 30 million hectares within a four year period (Figure 1). Herbicide tolerance has been the major trait as measured by area grown and this has largely been soybeans with tolerance to glyphosate herbicide, although other crops and other herbicide tolerance traits increased in 1999. Approximately one-third of the US commercial transgenic area is insect-protected covering maize, cotton, and a small area of potatoes. In each case, the protection was against herbivorous lepidopteran or coleopteran insects and has been conferred by using a modified form of one of the many genes that code for *Bacillus thuringiensis* (Bt) natural endotoxin proteins.

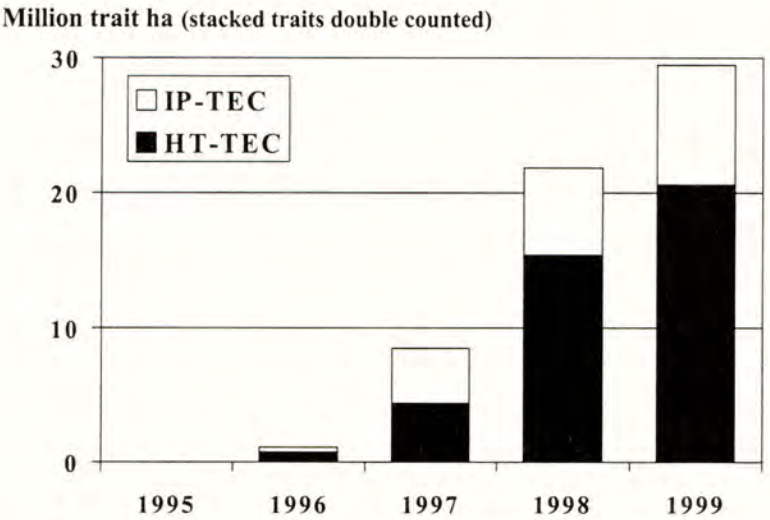


Figure 1. Estimates for the trait hectares of commercial US transgenic crops. Trait hectares are distinct from physical hectares because gene stacking in a crop results in two transgenes on the same hectare = 2 trait ha. In 1999, the US physical hectare area that contained transgenes was 27 million ha. IP-TEC and HT-TEC refer to "insect protected" and "herbicide tolerant" trait enhanced crops, respectively.

Are commercial HT-TECs worthwhile?

Weed control programs already exist for most major crops and each has a set of strengths and weaknesses. For HT-TECs the advantages arise from the technical and commercial features of the particular herbicide to be used with the crop, and how those features compare with the best alternate programs available. Approved HT-TECs include glyphosate and glufosinate tolerance in a number of crops, and bromoxynil tolerance in cotton. The largest current commercial situation in the US is glyphosate tolerant soybeans, which were grown on 15 million hectares in 1999, representing just over 50% of the total US soybean crop. Thus, as an example, it is worthwhile comparing recent glyphosate programs to previously available herbicides.

Glyphosate is a well-characterized post-emergent, systemic, non-selective herbicide with activity against a broad range of weed species (Grossbard & Atkinson, 1985). Technical details of the significant multi-year program that led to the successful development of transgenic glyphosate tolerance have been reviewed (Padgette *at al.*, 1996). Commercial experience on millions of hectares has clearly demonstrated that several advantages exist for HT-TECs, and for glyphosate tolerance in particular:

Crop safety is excellent because it has been designed and built-in. Herbicides are often a compromise between weed control and lack of crop phytotoxicity. In conventional situations, a practical level of selectivity is achieved by chemically altering candidate active molecules to reach a "best fit" for the crop. In some cases, increased crop tolerance to particular herbicides has been achieved by applying chemical selection pressure within a population of chemically-induced mutants. The best mutant line is then back-crossed into the crop germplasm base: for example, as with sulfonylurea tolerance in soybeans (Saari & Mauvais, 1996). With HT-TECs, improved crop safety arises through the specific addition of a gene whose product either degrades the herbicide or inhibits the binding site reaction – allowing the crop to be "fitted" for the best herbicide candidate.

Pre-emergent, soil applied herbicides are typically used as a prophylactic treatment while post-emergent herbicides can be used as required: providing, at least, the opportunity for lower chemical loading into the environment. Glyphosate tolerant soybeans have demonstrated that in many cases a single application of glyphosate can replace multi-herbicide programs. The availability of glyphosate tolerant soybeans has also been a major driver in the movement towards less tillage. Consequently, there has been a dramatic decrease in soil erosion with the obvious associated environmental benefits.

In terms of economics, the effectiveness of glyphosate tolerant soybeans is such that typical herbicide costs are often decreased by 30-50% per unit area. In addition, improved crop safety provides the opportunity for more yield per unit area, and at a lower input cost. In addition to enhancement of the direct economic returns, the parallel environmental benefits provide for significant indirect returns via improved soil and water management.

Why use IP-TECs in commercial practice?

Commercial IP-TECs currently only involve the use of Bt genes that encode insecticidal "Cry" proteins (McLaren, 1998). In the US, in 1999, various *cry* genes were used on about 7-8 million ha of maize and about 2 million ha of cotton, to control lepidopteran insects.

In maize, the main target pest is the European Corn Borer (ECB), *Ostrinia nubilalis*. After over-wintering as cold-tolerant larvae, the moths emerge in Spring, mate within a few days, and lay egg masses on young maize plants. Hatching larvae move into the whorl and begin feeding on the developing leaves. Eventually these larvae crawl down the plant and begin to burrow into the stalk creating feeding tunnels. These damaged plants yield less and are more susceptible to harvesting losses due to lodging, or premature ear-drop. While in some severe infestations insecticides are used to diminish the damage, the larvae are typically inside the maize whorl or stalk and may never receive sufficient exposure to the insecticide. Transgenic Bt maize is an excellent approach for ECB since the larvae are exposed as they eat into the developing leaves and maize stalk. Feeding larvae are controlled before they can cause

significant tunneling damage. Yield advantages for Bt maize vary from 200 to 2,500 kg/ha depending on the severity of insect attack, and the local conditions in each field.

ECB moths that emerge in mid-summer will mate and each female will lay about 400 eggs. These second generation larvae will feed on pollen, collar tissue, and may bore into the developing ear. The tunnels allow secondary infestations by fungal pathogens that often result in the formation of dangerous mycotoxins, such as aflatoxin or fumonisin (Figure 2). Lower insect damage can result in dramatically lower mycotoxins which is a major improvement in feed and food safety.

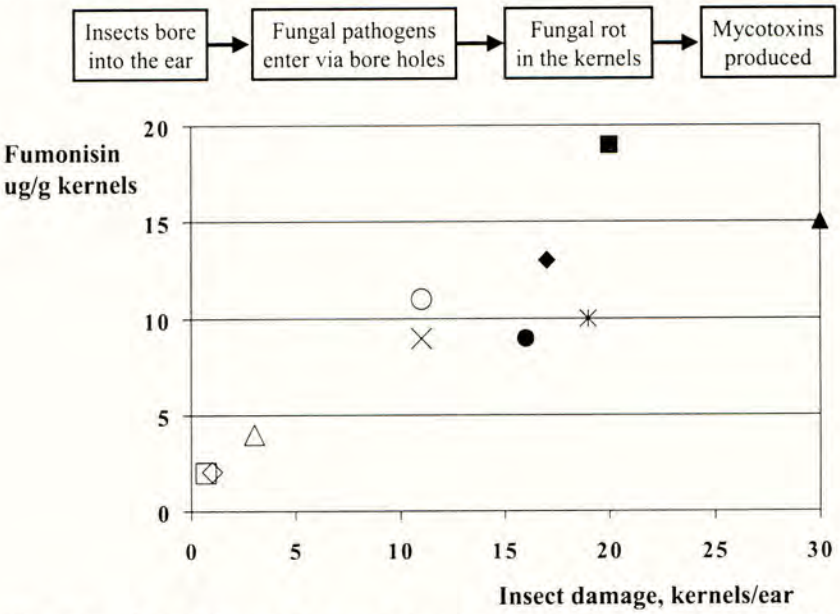
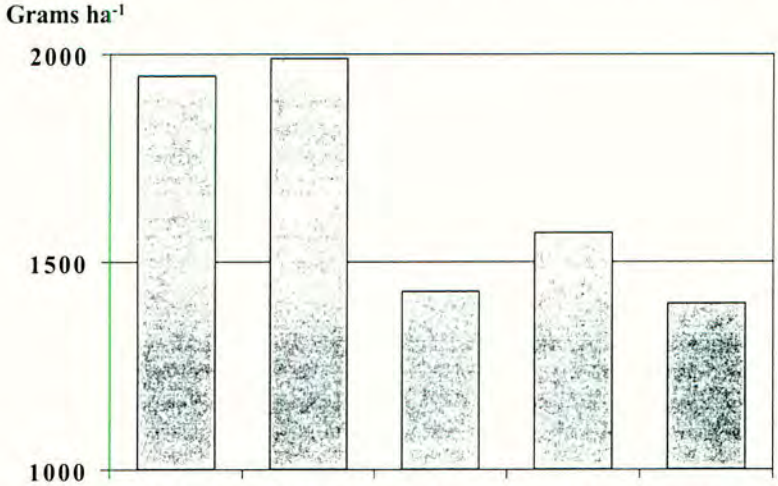


Figure 2. The relationship between insect damage and fumonisin levels from a 1997 field experiment in maize. Each symbol type represents a pair of near isogenic hybrids from a particular seed company, the open symbols are the transgenic Bt version and the solid symbols are the regular hybrid. Original data extracted from Munkvold and Hellmich (1999).

In cotton, Bt genes are used for protection against several insects that destroy the flower buds or cotton bolls. The cotton bollworm (*Helicoverpa zea*) and tobacco budworm (*Heliothis virescens*) complex infests 3.5 to 4.0 million ha. A single cotton plant may be host to 1,500 eggs with devastating results when they hatch. A series of insecticides have been used over time, as the insects developed resistance to each chemical class: calcium arsenate in the early 1900s; organochlorines in the 1940s; organophosphates in the 1960s; and a series of different pyrethroids since the late 1970s. Today, the conventional approach to insect control is to spray insecticides 4 to 8 times on each crop.

Use of the Bt *cry* 1A(b) gene in cotton offers protection against these major herbivorous insects with increases in lint yield that range up to 30%, depending on the local conditions, insect severity and insecticides applied. The net economic advantage, taking into account all the costs and benefits, is often in excess of $80 per ha. In addition to yield protection, the use

of Bt cotton results in large decreases in the number of insecticide applications per year, and in the total amount of insecticide applied per unit area (NASS, 1999). Figure 3 shows data for the pre-Bt cotton era and following commercial adoption in 1996. During recent years the total planted area for cotton has increased. Comparing the average for 1994/95 with the average for 1997/98, it can be estimated that Bt cotton has saved 11 million sprays per year. This environmental advantage translates into over 2470 tonnes less insecticide applied per year.



	1994	1995	1996	1997	1998
No. sprays ha^{-1}	4.3	5.0	1.8	1.8	1.9
Ha planted (Million)	4.0	4.7	4.8	5.3	4.9

Figure 3. Average amount of cotton insecticide applied per year over the period for Bt cotton adoption, in major cotton States. The number of applications per ha and the total ha planted are also shown. Data from NASS (1999).

One of the issues with insect control is that resistance can develop when the population is placed under selection pressure, as has happened in response to insecticides. With the use of Bt genes there is a requirement for management plans that mitigate the rapid development of insect resistance. One basic rule in this plan, for both cotton and maize, is the use of refugia: defined as an adjacent area where the Bt genes are not used, and it may or may not be protected by the use of a different mode-of-action insecticide depending on the situation. For Bt maize in the US Corn Belt, a minimum of 20% structured refugia is required, while for Bt maize in the Cotton Belt a minimum of 50% is required (EPA, 2000). The concept is that having an area free of insect control will maintain a minimal level of susceptible pests that can reproduce with any adjacent surviving resistant insects, thereby diluting any resistance genes in the next generation insect population. Various estimates of the impact of refugia have been made, with the expectation that problematical insect resistance may be delayed by 30 to 100 years.

The commercial trait enhanced crops available today have crop protection traits that have advantages in terms of production systems, and efficiency. However, it is also clear, and often under-estimated, that additional benefits arise from these TECs in terms of environmental advantages.

FUTURE AGRONOMIC TRAITS

Much of the promise of crop biotechnology is still in the R&D stages and commercial impact lies in the future. Recently, a large number of predictions have been focused on the future benefits to the consumer from direct use of enhanced composition and health attributes. While these quality traits are in the pipeline, we should not forget the potential agronomic traits that will provide indirect benefits to the consumer, coupled with direct potential benefits to the environment. The agronomic trait types being evaluated in field trials in the US have changed in relative proportion in recent years. While herbicide tolerant and insect protected crops remain of interest, the disease and crop agronomy type traits have increased such that 1999 alone was equivalent to the first ten years of testing (Figure 4).

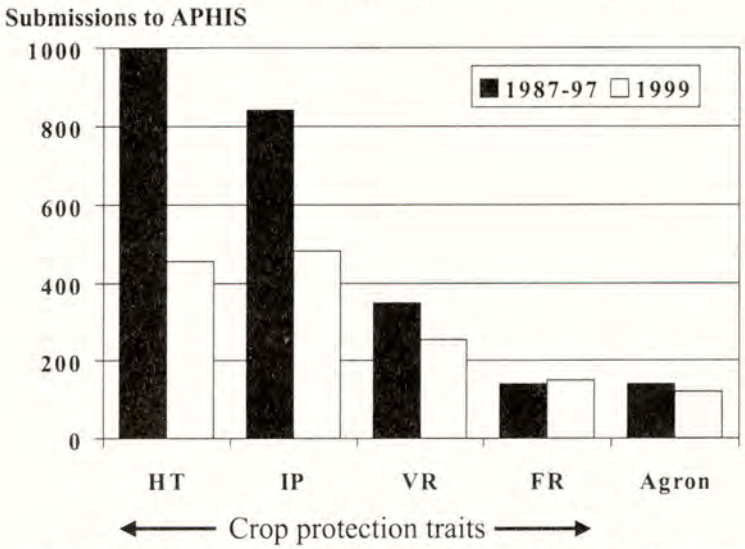


Figure 4. Average Analysis of regulatory submissions to APHIS for transgenic field trials, showing totals for the first 10 years (1987 to 1997) compared to 1999 alone. HT = herbicide tolerant; IP = insect-protected; VR = viral resistant; FR = fungal resistant; Agron = agronomic traits but excluding crop protection traits. Data from APHIS (2000).

Within the 1999 field evaluations there were a diverse number of single trait events, as well as many stacked trait events. The trait types being tested are listed in Table 1. HT-TECs include tolerance to a broader range of herbicide actives. IP-TECs cover several new insect pests and include some near commercial evaluations for maize rootworm control – a particularly damaging pest that causes over $1,000 million of damage in the US Corn Belt. Many new crop protection traits have been introduced covering fungal, viral, and bacterial diseases. Should these reach commercial products then it can be expected that the losses of

potential yield to pests and diseases will decrease significantly, as will use of the synthetic chemical protectants required for conventional crops.

Table 1. Crop protection and other agronomic type traits that are currently undergoing field testing in the US. The trait descriptions are as documented in the regulatory submissions (APHIS, 2000).

Herbicide tolerant	**Insect resistant**		
Bromoxynil	Aphids		
Chloroacetanilide	Coleopteran beetles (Colorado potato beetle, rootworm)		
Glufosinate	Leaf roller		
Glyphosate	Lepidopteran caterpillars (ECB, bollworm, armyworm)		
Imidazolinone	Sod webworm		
Isoxazole			
PPO inhibitor	**Fungal resistant**	**Agronomic**	
	Alternaria	Aluminium tolerant	
Virus resistant	Anthracnose	NH_4 assimilation	
Barley yellow dwarf	Apple scab	Carbon fixation	
Closterovirus	*Botrytis*	Growth rate increased	
Cucumber mosaic	Brown spot	Hormone level	
Gemini	Dollar spot	Male sterile	
Nepovirus	Ear mold	Nitrogen metabolism	
Potato leaf roll	*Fusarium*	Photosynthesis	
Potato virus X	Grey leaf spot	Plant development	
Potato virus Y	*Helminthosporium*	Senescence delayed	
Tobacco rattle	*Phytophthora*	Seed weight increased	
Tomato spotted wilt	Powdery mildew	Stalk strength	
Watermelon mosaic 2	*Rhizoctonia*	Yield increased	
Zucchini yellow mosaic	Rice blast		
	Rust	**Stress tolerance**	
	Sclerotinia	- drought	
Bacterial resistant	*Septoria*	- heat	
Bacterial speck	Smut	- oxidative	
Crown gall	White mold	- salt	
Erwinia	*Verticillium*	- water	
Pseudomonas			
Xanthomonas	**Other**		
	Metabolism of halogentaed hydrocarbons		
	Mycotoxin degradation		
	Root-knot nematode resistant		
	Systemic acquired resistant altered		
	Wound response altered		

In addition to the promise of improved crop protection, there are many traits directed at improving the efficiency of crop production including plant growth and development. In the 1970-80s there was a large scientific effort to discover and develop plant growth regulators (PGRs) to improve production. With the exception of a few growth retardants, this industry-wide PGR program really failed against expectations. It may be that the focus was on growth when, in fact, it is plant development that impacts crop production more. Development appears to be a sequence that involves gene sets switching on and/or off – difficult to achieve using an externally sprayed chemical PGR. Regulation of endogenous genes may provide a new opportunity to attempt the goals of the previous PGR programs but with new and more precise science.

Several plant stress related events are under evaluation with the promise of extending the growing season, shifting geographic limitation zones, and preventing loss to unexpected conditions such as late frosts. An interesting aspect of stress tolerance traits may be to allow crops to be grown on compromised land: e.g. high salt build-up due to years of irrigation can destroy soil fertility, but this could be utilized, if not rejuvenated, via biotech crops.

Nitrogen-use efficiency improvement could make a large environmental impact via the total applied volume and by decreasing potential run-off into streams and lakes. Phytoremediation using transgenic crops is also under evaluation as a possible mechanism for environmental enhancements.

Focused on the longer-term needs, there are many research projects exploring the utilization of plants as bio-factories (McLaren, 2000b). The provision of renewable resources will be critical as the existing finite fossil fuel pool continues to decline. If raw industrial inputs, and other renewables such as bioplastics, can be produced from plants then continuing to increase the level of output and protecting crops from the ravages of pests and diseases will becoming increasingly important.

CONCLUSIONS

For traits that alter composition in a value-added manner there will be a need to separate the crop output, called "identity preservation." For most crop protection and other agronomic traits there is no logical need for identity preservation: the harvested part is either increased in amount or protected from loss due to pests and diseases and is not changed in any substantial compositional manner. Thus, agronomic traits can provide many advantages, as described above, related to efficiency and environmental benefits, without the need to preserve the identity of the harvested output. From this perspective, agronomic traits may actually be more valuable to global production than more costly quality traits. A more open acceptance of scientifically-based crop production methods would help realize the potential efficiency of agronomic traits by removing the imposed "GM" handling and marketing costs.

Crop production will continue to improve through the utilization of new science and technology. Some developments will be in computerized equipment, informational agents, and in land management through satellite-based GIS/GPS advances. However, specific genetic alteration rather that conventional genetic selection will play a major role in future progress. In the free economy agricultural systems, food and feed prices at the farm-gate have remained relatively static for many years. Yet, a sophisticated food manufacturing

system has developed that captures large financial gains from fixing a basic problem with its own inputs – plants are still composed for reproduction, not designed to be eaten. In the final analysis, it may be that what the food processor is willing to pay, what is accepted as a reasonable margin, and how much is passed on to the consumer, will determine the relative outcome of future trait types.

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Resistance to Plant Diseases

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ABSTRACT

Systemic Acquired Resistance (SAR) is a response plants make to pathogen invasion which potentiates active defence. Considerable research is directed toward defining pathways that eventually lead to resistance, and the role of salicylic acid, jasmonates and ethylene in these events. Probenazole and acibenzolar-S-methyl are two commercially available products identified by conventional screening, and which show that the concept of chemical manipulation of SAR can be viable. More sophisticated *in planta* screening systems offer ways to identify novel chemistry with fewer adverse effects on crop growth by avoiding, for instance, unwanted activation of hypersensitive necrotic reactions. But the full potential of chemical plant defence activators can only be exposed in field experiments, where the sometimes slow response of SAR can be augmented with conventional fungicides, at perhaps reduced rates, to produce durable and acceptable disease management systems.

INTRODUCTION

Molecular technologies have transformed approaches to defining mechanisms of resistance to disease in plants. Structural genomics has generated vast amounts of sequence data which indicates that at least 1%, and possibly more of these genes in *Arabidopsis thaliana* and rice are involved in disease resistance (Michelmore, 2000). Comparative genomics, linked with high throughput mapping techniques, reveals that many of these genes are clustered, and that the majority of the 20 or so resistance genes cloned so far have leucine-rich repeats at their N-terminal-end. Functional analysis of these genes is only just beginning, but should eventually lead to designing resistance genes so that their products recognise invading pathogens, and induce an appropriate response. However, where major genes for resistance are involved these often only protect against one race of a pathogen, and there is a long history in many crop species of the selection of new pathogen races that overcome resistance. It remains to be seen if a molecular approach to deploying these major resistance genes will be any more durable than conventional plant breeding.

There is no doubt that plants deploy mechanisms, other than those involved in "gene for gene" interactions, to defend themselves against attack by fungi, bacteria and viruses. A key defence component induced by pathogens spreads in some, as yet unknown way, throughout the host, and is called Systemic Acquired Resistance (SAR; Ross, 1961). Primary infection with a necrotizing pathogen potentiates a plant defence response to any secondary infection. SAR is associated with a co-ordinate expression of a series of genes, including those encoding some pathogenesis-related (PR) proteins, and reduced penetration and lesion formation by the challenging

pathogen (Hammerschmidt, 1999). Normally susceptible plants have the potential to develop SAR and become resistant, but only when their defence response is quick enough to outpace the pathogen. Although SAR is induced by action of pathogens, this effect can be mimicked by many synthetic molecules (Table 1). The availability of loss-of-function mutants, especially in *Arabidopsis*, has revealed the outline of signalling pathways involved in SAR, but a largely empirical screening process has already produced two commercial products that control disease through activation of host-plant resistance.

Probenazole (Oryzemate; Watanabe *et al.,* 1979) has been used in Japan for more than 20 years to control rice blast (*Magnaporthe oryzae*) and bacterial sheath blight (*Xanthomonas oryzae),* although it has no direct effect against these pathogens, and instead triggers defence reactions in the host. More recently, acibenzolar-S-methyl (Bion; Goerlach *et al.,* 1996) has been introduced to augment control of fungal, bacterial and viral diseases in a wide range of crops. Despite intensive use over many years the performance of probenazole has remained unaltered, suggesting that this approach to disease control is durable. Probenazole and acibenzolar-S-methyl clearly prove the concept that chemical activation of host defence mechanisms is a viable way to control a wide range of plant diseases, with seemingly little adverse environmental impact. Our paper emphasises the SAR aspect of disease resistance.

Table 1. Some chemistry that activates SAR

Commercial products

Compound	Common name	Diseases controlled
3-allyloxy-1,2-benzisothiazole-1,1 dioxide	Probenazole	Rice blast Rice sheath blight
Benzo(1,2,3)thiodiazole-7-carbothioic acid S-methyl ester	Acibenzolar-S-methyl (BTH)	Fungal, Bacterial, Viral

Experimental compounds

Compound	Comments	Diseases controlled
1,2 benzisothiazole-3(2H)-one 1,1 dioxide	Metabolite of probenazole	Rice blast
2,6 dichloroisonicotinic acid	INA	Fungal, Bacterial, viral
Methyl jasmonate		Bacterial
1-aminocyclopropane-1-carboxylate	Ethylene precursor	Bacterial
Acetyl salicylate	Aspirin	Viral

KEY FEATURES OF SAR

Plants respond in many ways to attempted invasion by pathogens. One response that can occur within minutes of infection involves a rapid increase in Reactive Oxygen Species (ROS; the so called "Oxidative Burst", Lamb & Dixon, 1997), which may directly kill pathogen cells, or stimulate lignification confining the pathogen to its initial infection site. These changes are often associated with a hypersensitive response. Subsequent responses are slower, and may take several days before the full extent of SAR is achieved through PR protein expression. The biological features of SAR are summarised in Table 2.

Table 2. Biological features of SAR

1. Induction by pathogens, chemicals or abiotic stress.
2. Several days elapse between induction and full expression.
3. Protection conferred on plant tissues not challenged at infection.
4. Effect is seen as fewer, smaller lesions, and a reduction in pathogen multiplication and sporulation.
5. Protection lasts for weeks and even months.
6. The systemic signal is graft-transmissible but is not passed on to seed.

Adapted from Lucas (1999)

A key intermediate in many SAR responses is salicylic acid (SA) which induces non-specific expression of many defence-related genes (Dempsey *et al.*, 1999). Transgenic *A. thaliana* and tobacco plants expressing salicylate hydrolase (*nah*G gene) fail to accumulate SA or PR proteins, and are particularly susceptible to pathogens that normally induce resistance (Delaney *et al.*, 1994). Salicylic acid can directly inhibit catalase and ascorbate peroxidase (Rao *et al.*, 1997), which normally scavenge for ROS and counter the anti-microbial effects of any oxidative burst. Indeed, inactivating these enzymes generates free SA radicals (Kvaratskhelia *et al.*, 1997), and these may themselves activate host defence mechanisms. In many plants SA also induces expression of alternative oxidase (AOX) above constitutive levels, and this correlates well with localisation of virus into discrete lesions (Murphy *et al.*, 1999). How AOX is involved in SAR is far from clear, but its effects are restricted to virus control, and not other pathogens. Salicylic acid also mediates in several signalling pathways (Dempsey *et al.*, 1999) which eventually lead to the expression of PR proteins, such as chitinases and glucanases, which have anti-fungal activity.

In some cases SAR occurs in *nah*G *A. thaliana* plants indicating that defence pathways can be activated independently of SA. Furthermore, defence responses are not always linked with increases in SA levels. Plant growth regulators, jasmonic acid and ethylene are important signalling molecules in these alternative pathways (Pieterse & van Loon, 1999). Induced systemic resistance can also follow wounding, for example after insect attack, and this response is mediated through jasmonates and ethylene. Induction leads to the expression of PR proteins, many of which are also induced by SA. But these SA-independent pathways generate resistance to a different spectrum of diseases than operates following SA induction. Depending on the invader, plants appear capable of switching on whichever pathway is appropriate, or

indeed several. Cross-talk between salicylic acid, jasmonates and ethylene pathways offer great regulatory potential, and the results may be synergistic (Xu *et al.*, 1994; Lawton *et al.*, 1994), or antagonistic (Thaler *et al.*, 1999).

SCREENING FOR COMPOUNDS THAT ACTIVATE SAR

Given the emphasis that surrounds SA in research on defence pathways it is perhaps not surprising that some of the active chemistry focuses around benzoic acid derivatives (Table 1). The two commercial products emerged from conventional screening protocols although, of course, these were confined to *in planta* assays. Fortunately SAR seems to follow common themes throughout higher plants, although there may be differences in detail between monocot and dicotyledonous crops. Consequently, a model system using *A. thaliana* and *Peronospora parasitica* (downy mildew) has been a "first–step"screening tool prior to using crop plants and priority target pathogens. As more detail emerges about how SAR is implemented, sophisticated screens can be developed using transgenic plants to target key steps in defence pathways. Blocking SAR pathways through deletion, disruption or anti-sense have all been used to generate susceptibility in otherwise resistant host-pathogen combinations. Screening can then be carried out to identify compounds that have no direct action against the pathogen, but which restore resistance and control disease, by enhancing downstream steps in the SAR pathway. Coupling the promoter sequences of genes involved in SAR with a reporter system such as GUS, luciferase, GFP or herbicide resistance, provides transgenic plants that can be used to screen for compounds that enhance gene expression and control diseases. SA, and other signal compounds involved in SAR, only potentiate the defence response which is then triggered by exposure to an elicitor produced by the pathogen. SAR may take several days to develop, so not only is careful timing needed for challenge inoculations, but choice of pathogen may be crucial to identify lead compounds.

FIELD PERFORMANCE

The diversity of factors that activate induced resistance includes many environmental stimuli that influence crop growth, such as drought stress, damage caused by pollutants, and wounding following insect attack. The many micro-organisms in the phylloplane may also initiate a defence response. These all augment any SAR induced by chemical activators so, contrary to the common experience with conventional fungicides, performance of development compounds in the field can be better than predicted from greenhouse screens. Effective SAR results from a matrix of interacting pathways, some of which may generate additional benefits, such as insect resistance (Bostock, 1999). However, any extensive necrosis accompanying activation of the hypersensitive response greatly reduces photosynthetic capability and yield. More specific targeting of steps in the defence pathway which only affect pathogen development may avoid some of these undesirable side-effects. For some diseases, protection afforded by activation of SAR may be insufficient to achieve commercially acceptable levels of disease control, and field evaluation should explore the benefits of any new chemistry within the framework of conventional fungicide use. For example, acibenzolar-S-methyl improved significantly control of post-harvest rots of melons when given as a pre-flowering spray, and combined with a post harvest dip in

guazatine (Table 3; Huang *et al.*, 2,000). By itself acibenzolar-S-methyl gave unacceptable performance.

CONCLUSIONS

Chemical manipulation of SAR offers considerable potential for durable disease control strategies with little adverse environmental impact. Success undoubtedly requires improved understanding of the various defence pathways, and the interplay between them, so that undesirable phytotoxicity and effects on crop development can be avoided. Access to the many molecular genetic technologies has opened the way to define SAR pathways, in ways that could not be achieved through conventional genetics and biology alone. But evaluation of defence activating compounds under field conditions is essential, not only to record levels of disease control, but to identify diseases where they can make most impact. This includes exploring disease control strategies that that combine SAR compounds with conventional fungicides with the aim of achieving acceptable disease control using lower fungicide rates. Defence activating compounds should have a useful role in Integrated Disease Management, with the benefit of reducing selection for resistance to fungicide partners. Above all, success will rest with the ability of growers to learn how to optimise use of these new tools within their disease management systems.

Table 3. Effect of acibenzolar-S-methyl and guazatine in rockmelons after storage *

Treatment	Percentage of fruit **			
	Diseased	Infected by		
		Alternaria	*Fusarium*	*Rhizopus*
Control	100.0a	92.3a	33.3c	21.7d
Acibenzolar-S-methyl	72.6c	62.0b	7.6e	7.6e
Guazatine	98.0b	98.0a	4.1ef	2.0f
Acibenzolar-S-methyl plus guazatine	2.0d	0.0g	0.0g	0.0g

From Huang *et al.*, 2000

* Acibenzolar-S-methyl (50mg/l *a.i.*) applied as a foliar spray before flowering. Guazatine (500mg/l *a.i.*) applied as a post-harvest fruit dip. Melons were stored at 2-8 C for 3 weeks and a further 2 days at room temperatue.

** Figures followed by the same letter in the first column, and separately in the last three columns , were significantly different at 5% by LSD

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Transgenic baculoviruses

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The transfer of traits to wild relatives

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ABSTRACT

The possibility of transferring traits from genetically modified crop plants to wild relatives is regarded as a key issue in the commercial introduction of GM crops. It is often presented as a vague and uncontrollable threat to the wider environment. In fact, gene introgression may be restricted to a few species, be local in nature, and face a range of biological barriers. Studies of wild and feral *Brassica* populations are used to illustrate how gene flow may be quantified and the consequences for environmental impact assessed.

INTRODUCTION

The potential movement of transgenes by hybridisation between crops and their wild relatives has long been regarded in Europe and the UK as a key issue in the commercial development of genetically modified crops. Highlighted in the report of the Royal Commission on Environmental Pollution in 1989 as "an important uncertainty" (in risk assessment) (RCEP 1989), the possible spread of novel genes in the environment has remained a major focus of concern in the GM debate. The possibility of widespread genetic "pollution" and the creation of "superweeds" has been a central plank in the anti-GM stance of several pressure groups (*e.g.* Greenpeace (Fronwald & Strauss 1998), Genewatch 1998), measures to restrict gene transfer by mechanisms such as plastid transformation, suicide genes and other gene protection systems are an increasingly important part of the developing technology, and there has been a burgeoning growth of research on the measurement and consequences of gene flow - much of it summarised in an important BCPC symposium last year (BCPB 1999). Contributing to that meeting, Hill traces a quickening of the debate in the UK to the first application, in 1994, for a commercial release of oilseed rape (the (then) PGS hybrid and herbicide-tolerant line) (Hill 1999).

The transfer of traits from crop to crop is generally regarded as either an agronomic problem or one of product segregation. There is much official optimism that the management of volunteers and the separation of "GM" and "non-GM" food can be achieved by good practice and agreed thresholds for the minimum adventitious presence of GM material in non-GM food (*e.g.* the 3rd Report from the House of Commons Agriculture Committee, Session 1999-2000 on "The segregation of genetically modified foods"). By contrast, the transfer of traits from crop to wild relative is often presented as a rather vague, unspecified, unquantifiable, uncontrollable and irreversible ("genie out of the bottle") threat to the wider environment. But is that appropriate?

TRAIT TRANSFER - BACKGROUND

As indicated above, much has been written about gene flow. Here, I make three points which I believe tend to be overlooked.

The first point is that we are actually dealing with relatively few plant species. Not only do crops in general comprise a tiny subset of all plant species but the development of modern agriculture has led to many of these crops being grown outside the geographical range of their wild relatives. Although Ellstrand *et al.* (1999) point out that 12 of the world's 13 most important food crops (by area harvested) hybridise with wild relatives, their review reveals that hybridisation is frequently restricted to a small part of their distribution. Further, whilst all 12 produce spontaneous hybrids, ranging from fertile to fully sterile, and intermediate phenotypes, evidence from genetic and molecular studies of extensive introgression is limited to six of these (rice, cotton, sorghum, pearl millet, rapeseed and sunflower). Whilst there is good evidence of hybridisation in some crops, such as beet, grain *Chenopodium* and squash, and less evidence but good biological reasons to suppose it occurs in many others (such as ryegrass and white clover), for a very large proportion of crops in many parts of the world gene flow is simply not an issue. Thus, in the UK, GM maize, potatoes, tomatoes, wheat, peas and beans do not present a risk of trait transfer to wild relatives (see Raybould & Gray 1993). In summary, the problem of transgene transfer can be targetted onto relatively few species.

Secondly, it must be assumed that for many crops the exchange of genetic material with their wild antecedents and relatives has been a long process, dating back to their early domestication. For example, Renno *et al.* (1997) show that cultivated and wild forms of pearl millet (*Pennisetum glaucum*) have exchanged genes for at least 3,000 years, co-evolving over large parts of the Sahel. Indeed, introgression between wild and cultivated plants is notoriously difficult to prove in cases where there are "crop-weed-wild species" complexes, where convergent evolution has produced crop "mimics", and where common ancestry results in shared traits. Where modern cultivars continue to exchange genes with wild relatives, the latter are frequently agricultural weeds or species with similar life history (see *Brassica* example below). There is no *a priori* reason to suppose that transgenes will behave differently in this situation from other genes, or that transgenic crops present a greater threat of genetic erosion in centres of diversity than their conventional counterparts. The likelihood of trait transfer must be considered separately for each crop-wild relative relationship.

Finally, we should observe that in the current GM lexicon "gene flow" has become a shorthand term for trait transfer, including hybridisation and introgression. This is close to its meaning in its population genetic sense ("the incorporation of genes into the gene pool of one population from one or more populations" (Futuyma 1998)), but cross-pollination, and even pollen transport, are frequently used interchangeably with gene flow. It is important to remember the barriers to introgression which follow pollen movement - lack of coincident flowering, local pollen competition, poor pollen viability or pollen tube growth, lack of zygote formation, low or selective post-zygotic seed maturation, low relative F_1 seed survival, and so on). The "if it can happen, it will happen" philosophy which, perhaps necessarily, underpins risk assessment may give a hugely unrealistic estimate of the magnitude of actual trait transfer.

TRAIT TRANSFER - HOW MUCH?

Where the distribution of a crop overlaps with a wild conspecific with which it is interfertile, the assumption must be, as indicated above, that gene flow and introgression will occur. Thus, in the UK the risks of growing transgenic crops of beet, ryegrass, carrots, cabbage, white clover and lucerne (and trees such as apple, plum, poplar and Scots pine) will include

transgene transfer - and the risk assessment must address the possible consequences (the "So what?" question), rather than attempt to assess the likelihood. For other crops, however, especially those with congeneric or closely-related wild relatives, the probability of introgression is an important issue. The classic example in European agriculture is oilseed rape.

The possibility of transferring transgenes to the wild relatives of oilseed rape, the first crop to be approved for commercial release in the UK, has received a great deal of attention. The ease with which hybrids can be made between oilseed rape (*Brassica napus*) and the closely-related, mostly diploid, species in the Brassicaceae family varies from spontaneous hybridisation to a requirement to cultivate ovules or embryos in the laboratory. The burgeoning research in this area has revealed six wild species with which spontaneous hybridisation (the production of hybrids by natural pollen transfer unassisted by man) can occur: *Brassica rapa*, *B. oleracea*, *B. juncea*, *Hirschfeldia incana*, *Raphanus raphanastrum* and *Sinapis arvensis*. Although 11 other species have produced hybrids by manual pollination and/or embryo rescue (Gray & Raybould 1999), the threshold of spontaneous hybridisation can be accepted as appropriate for the assessment of gene flow occurring under field conditions.

Table 1 **Gene flow between oilseed rape and wild relatives (from Gray & Raybould 1999)**

Species	Spontaneous hybridisation				Gene flow
	F	M	BC	F_2	
Brassica rapa (2n = 20) Wild Turnip, Bargeman's Cabbage, Navew	✔	✔	✔ *	✔	High when small numbers of *B. rapa* in oil-seed rape field. Hybrids of backcrosses intermediate fit-ness. Introgression dependent on genome location, but probably rare and erratic in natural populations.
Hirschfeldia incana (= *Brassica adpressa*) (2n = 14) Hoary Mustard	✔ *	✔ ^{s}	✔	X	Hybrids have low fertility. Introgression unlikely because of genome incompatibility.
Raphanus raphanis-trum (2n = 18) Wild Radish, Runch, White Charlock	✔ *	✔ ^{s}	✔ *	✔ ?	Hybrids produced in small numbers and have low fertility. Introgression difficult because of unshared genomes.
Sinapis arvensis (2n - 18) Charlock, Wild Mustard, Kilk	-	✔ * ^{s}	-	m	Introgression unlikely, but little information.
Brassica juncea (2n = 36) Chinese Mustard, Indian Mustard, Brown Mustard	✔	✔	m	m	Low numbers (3%) of hybrids. No data on field performance or introgression.
Brassica oleracea (2n = 18) Wild Cabbage, Sea Cabbage	✔ *	-	-	-	Details unknown. Introgression possible in theory because of parental C genome.

✔ * = From new data since Scheffler & Dale (1994); ✔ ^{s} = crossed to male-sterile oilseed rape; m = manual cross

The likely extent of introgression in the six species can be assessed from Table 1, which summarises the work to date on hybridisation and the production, fertility and fitness of subsequent generations and backcrosses. This ranges from a high possibility, in favourable conditions, of introgression from oilseed rape into wild turnip (*B. rapa*) to a very low probability in charlock (*Sinapis arvensis*), in which spontaneous hybridisation to male-sterile oilseed rape was observed relatively recently (Lefol *et al.* 1996). Preliminary results from an extensive survey of charlock populations and attempted reciprocal crosses between charlock and oilseed rape confirm the very low probability of transgene transfer to the species (Moyes *et al.* 1999).

The extent of hybridisation and introgression of genes from oilseed rape into wild turnip, where the hybrid has been known for a long time (*e.g.* Davey 1939), has been found to vary considerably, depending on the conditions. At one extreme, where a few plants of wild turnip (which is self incompatible) occur in fields of oilseed rape, they may produce high numbers of hybrid seed (and *vice versa* where rape occurs in turnip fields). The recent work of Jørgensen and her colleagues has shown remarkably similar levels of hybridisation to those reported nearly 40 years ago by Palmer for single wild turnip plants in swede fields (88% in Palmer (1962), 93% in Jørgensen *et al.* (1996)), and for small groups of one species in crops of the other (groups of four (Palmer) or 5 (Jørgensen *et al.*) plants of *B. rapa* giving 10% and 13% hybrids, and of *B. napus* giving 5% and 9% hybrids, respectively). At the other extreme, the work of Wilkinson and colleagues (Scott & Wilkinson 1998, Wilkinson *et al.* 2000) has indicated that hybridisation rates with populations of *B. rapa* found outside oilseed rape fields (where it is found along the banks of canals and streams - hence the popular name bargeman's cabbage) are extremely low, and coupled with the high mortality rates in such populations, this will make transgene introgression slow and uncertain.

The latter of these studies provides the first reasonable estimate of realised gene flow between a crop and a wild relative in the UK (Wilkinson *et al.* 2000). Using a combination of satellite imagery to locate oilseed rape fields in 1998, and the screening of sympatric *B. rapa* populations in 1999 for hybrids using flow cytometry to detect triploids and molecular analysis (SSR - PCR primers that yield amplification products specific to the A and C genomes - oilseed rape being AACC and turnip AA) to detect true hybrids, only a single hybrid with *B. rapa* was detected in a 15,000km^2 area of south-east England. No hybrids were found in clifftop populations of wild cabbage (*B. oleracea*) within the same area.

Such low rates of hybridisation to wild relatives, even when hybridisation levels can be high in laboratory or field conditions, suggest that trait transfer could be extremely slow, and that post-release monitoring and, where necessary, containment is a realistic prospect. Much, of course, will depend on the relative fitness of the wild relative with and without the transgenic trait.

TRAIT TRANSFER - SO WHAT?

That traits may be transferred from GM crops to wild relatives by hybridisation and introgression is an important aspect of the assessment of the risk which may be involved in commercialising such crops. But the fact, or possibility, of transfer is merely the first step - the risk assessment must (and in my experience, always does) include an evaluation of the hazards posed by such a transfer and of the consequences of those hazards being realised. In

short, the key question is the so-called "So what?" question, which addresses the potential environmental impact of gene flow.

The challenge of the "So what?" question is that it takes us into an area of uncertainty and variability, where the underpinning science does not fit easily into the probabilistic risk assessment applied to, say, the release of a novel agrochemical. We cannot use parameters such as exposure time, dose rate or dilution, and there are no precise (or even widely accepted general) definitions of environmental "harm". Environmental impact may be measured as an increase in the growth rate or persistence of wild populations, as a change in the species' composition of a semi-natural ecosystem, or in the relative abundance of non-target species. The relatively young science of ecology has achieved much in the understanding of patterns and processes, but prediction is in its infancy, and we must learn to work within the bounds of our understanding of natural systems (see also ACRE 2000).

Arguably the most important potential hazard is that the transferred trait could confer increased weediness on wild relatives compared to non-transgenic traits. It is critically important to understand the effect of any novel trait on the relative fitness of the plants which express it. But fitness, like weediness, is context-, habitat- and even site-specific. Does this imply that every trait in every crop and wild relative to which it might be transferred should be tested in every possible environment in which it might occur? Must all trials be on the scale of the early PROSAMO experiments (for example, Crawley *et al.* (1993) studied transgenic and non-transgenic oilseed rape in 80 treatments in 12 sites over three years, totalling 2,880 sets of demographic measures)? Clearly such large-scale experiments are impossibly expensive to do for all crops and might yield relatively little information useful for risk assessment in relation to the effort expended.

Fortunately, there are alternative, more targetted, approaches to risk assessment and, specifically, to addressing the question of changes in plant fitness. Below, I distinguish three types of study which can inform the risk assessment, illustrating them with examples from our own laboratory. These are the use of (i) targetted experiments, (ii) population modelling, and (iii) natural populations.

Targetted Experiments

A targetted approach to risk assessment was initially advocated by Linder & Schmitt (1994, 1995) and has been used by them to measure the effects of a transgene on plant performance during those life-history stages when the transgene is most likely to affect fitness. These have included glasshouse and field experiments to test the performance of transgenic oil-modified oilseed rape (and of hybrids between *B. napus* and *B. rapa*) on seed dormancy, germination cueing mechanisms and early seedling growth (Linder & Schmitt 1994, 1995; Linder 1999).

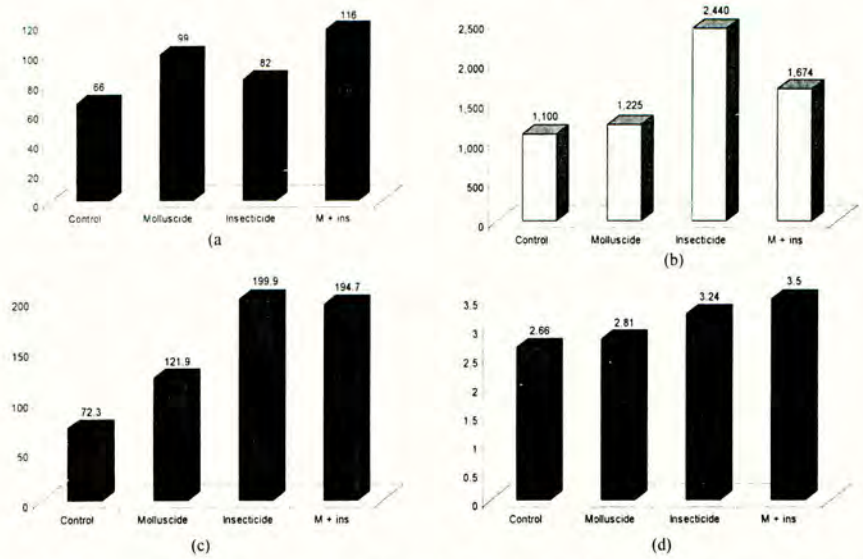
A different type of targetted experiment can be based on the "What if?" question. For example, an experiment described by Raybould *et al.* 1999 addresses the question "What if feral populations of oilseed rape were protected from invertebrate herbivory - would they become more persistent or invasive?" (The invasion and establishment of feral populations provides an alternative route for the escape of transgenes from agriculture.) Twenty-five experimental plots of spring oilseed rape variety "Aries" were sown at standard densities in a Latin square design involving three experimental treatments and a control. The treatments were periodic applications of molluscicide ("Draza"), insecticide (alternately "Hallmark" and

"Sybol"), or both. The plots were fenced to exclude rabbits and deer, and weed-free strips of 1.5m wide were maintained around each 2x2m plot. Plant density and seed output were recorded in July and August, seedlings were counted in the central plot and surrounding strips in the autumn, and the numbers of plants in each plot recorded the following year.

Average plant densities were increased under molluscicide treatment ($p > 0.05$), insecticide (non-significantly), and both ($p > 0.01$), the higher density in the insecticide treatment being due to reduced damage by flea beetles (*Phyllotetra* spp.) (Figure 1). Seed output per m^2 was

Figure 1

(a) No of plants/m^2 (b) seeds/plants (c) seeds/m^2 (d) seed dry weight



significantly different between treatments ($F = 6.28$, $p > 0.004$), mainly due to the effect of insecticide ($F = 19.52$, $p > 0.0001$), which chiefly reduced damage to flowers by pollen beetles (*Meligethes* spp.) and to seed by cabbage seed weevil (*Ceutorhynchus assimilis*). Density-dependent competition appeared to influence per plant seed production, with fewer larger plants occurring in the plots not protected against molluscs but protected against insects. In addition, significantly more seedlings were produced in the treatment plots than the control (although the numbers were small, with six seedlings in the controls (from around 250,000 seeds), 50 in insecticide treatments (from 750,000 seeds), 55 in molluscicide treatments (from 500,000 seeds), and 90 in insecticide + molluscicide (from 750,000 seeds). However, in the following year only 36 plants flowered in the whole experiment, 34 of these in one control plot presumably overlooked by deer.

The implications of this experiment are that transgenic insect-resistant feral oilseed rape plants will produce more seed than unprotected conventional varieties, but that other causes of seed and seedling mortality will prevent this increase in fecundity from leading to increased population growth rate or persistence. The observed causes of mortality included frost and vertebrate herbivores (pigeons, pheasants, rabbits and deer), but other unknown causes - particularly of seed loss in the soil - are likely. This suggests that, to increase population growth rate, in this context an appropriate measure of weediness, oilseed rape

requires a triat, or traits, which significantly reduces mortality between the stages of seed production and seedling establishment. Targetting experiments on the survival of seed in the soil and on germination is thus especially relevant (Linder 1999).

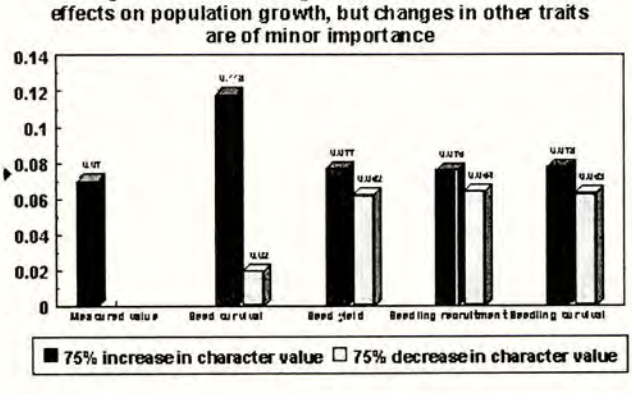
Modelling

Those life-history stages where changes in fitness may lead to increased weediness can also be identified using population growth models. Bullock (1999) has used population matrix models to ask which demographic parameters, if modified, will most affect weediness. In such models the demography of a particular population is represented by a stage projection matrix in which transitions from one life cycle stage to the next (*e.g.* seeds → seedlings, seedlings → adults, adults → flowering plants) are determined by combinations of the proportionate survival, the proportion that changes to a different stage, and the fecundity of the stage. Changes in abundance, *i.e.* the number of individuals in the population, from one year to the next are measured by λ the annual rate of population increase ($\lambda = N_t + 1/N_t$, where N_t = the number in year t, and $N_t + 1$ = the number in the following year. The stage projection matrix can be used to predict the effect on λ of changes in the life cycle by calculating the relative change in λ in response to small changes in one element in the matrix (say, increasing seed survival). This property of each transition is referred to as its elasticity (de Kroon *et al.* 1986) and provides a powerful way of directly modelling the changes in particular demographic parameter values and seeing how they affect λ.

Such models, as Bullock (1999) demonstrates, give insights into these demographic parameters linked to the weediness of particular species in particular, and relevant, habitat types. They are species-specific and habitat -specific (as is weediness, often mistakenly viewed as a general property of a species) and, when based on relevant experimental data, can be employed to highlight potential "high risk" modifications. For example, elasticity analysis of a stage projection matrix for oilseed rape, using data from an experimental population in Berkshire (part of the PROSAMO study), indicated that variation in seed survival had the most effect on the annual rate of population increase (Raybould *et al.* 1998). This effect can be seen in Figure 2, where the elasticity analysis has been supplemented by constructing separate matrices in which one parameter was either increased or decreased by 75%. λ was then calculated for each matrix and compared with the λ of the original matrix.

Figure 2

Outcome of modelling an Oilseed Rape population. Changes in traits affecting seed survival have dramatic effects on population growth, but changes in other traits are of minor importance



This mathematical experiment agrees with the field experiment described earlier in suggesting that any modifications which affect seed survival (as opposed to seed production) in oilseed rape have the potential to alter plant fitness. Modelling of this type can provide a framework for screening the effects of novel traits, and combined with other studies of plant fitness, help to target risk assessment on to the key questions.

Natural Populations

Ultimately, the spread of a trait in populations of the wild relative to which it has been transferred will depend on the relative fitness of plants with and without the trait. For many of the traits which are being engineered into crops, it is possible to ask whether there are functionally similar genes out there in the wild and, if not, what might be the effect of such a trait if it was transferred. Especially important in this respect are traits which are ecologically relevant, such as those which confer resistance or tolerance to pests and pathogens. For this reason it is important to increase our understanding of the rôle of specific pests or pathogens in regulating natural populations of crop relatives, and where genes which defend the plant against such stresses exist, to understand their distribution, dynamics and dispersal.

Of course, this is a huge subject area, even though, as outlined earlier, research can be targetted onto a few relevant crop/wild relative combinations. An example from our work on wild *Brassica* species may serve to underline both the complexity of the task and also, perhaps most important, the difficulty of providing generic risk assessments.

Figure 3
Frequency of six viruses in five wild populations of two *Brassica* species on the Dorset coast

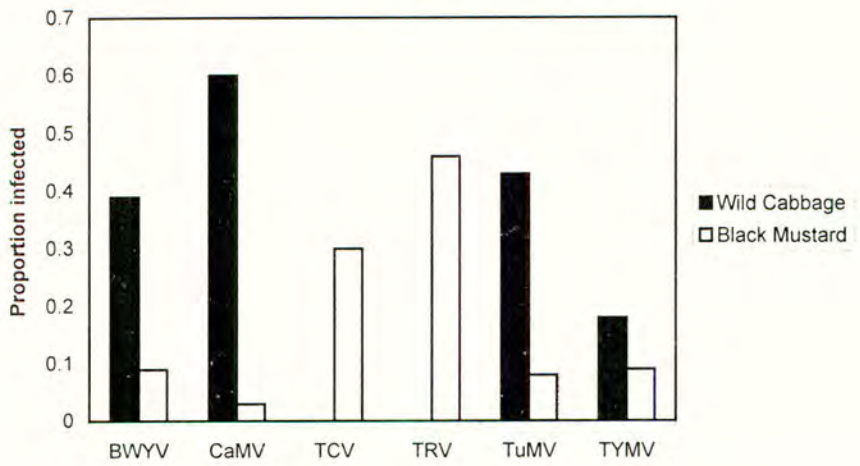


Figure 3 shows the frequency with which six viruses were detected in five wild populations each of two *Brassica* species (wild cabbage, *B. oleracea*, and black mustard, *B. nigra*) occurring along the Dorset coast (see Raybould *et al.* 1999 for location map). In some sites the two species occurred together - even so, viruses such as turnip crinkle virus (TCV) and turnip rosette virus (TRV) were not found on *B. oleracea*, and cauliflower mosaic virus (CaMV) and turnip mosaic virus (TuMV) were rare in *B. nigra*. Research by Dr I Cooper and Dr A F Raybould and their colleagues is building a picture of the relationships between

the viruses and the two hosts which is helping to explain these differences. For example, CaMV, present in 60% of all *B. oleracea* plants (and in 90% of plants in one population), had no significant impact on growth or fecundity compared to controls when inoculated into the plant at the three-leaf stage (M. Alexander, unpublished). By contrast, inoculation with turnip yellow mosaic virus (TYMV) killed 51% of an experimental population of 187 plants and reduced both average dry weight and seed production in the remaining plants to less than a third of that in the controls (Maskell *et al.* 1999). Interestingly, in view of TuMV's intermediate frequency in natural populations, inoculating plants with TuMV had an intermediate effect on mortality, no effect on growth, and a similar effect on seed production. Preliminary results on the impact of virus on *B. nigra* indicate that CaMV and TuMV and, perhaps unexpectedly, TCV and TRV have significant impacts on survival and fecundity (M. Thurston, unpublished).

These results demonstrate clearly that ecological risk assessment of transgenes for virus resistance must do more than survey adult plants in natural populations of wild relatives for the presence of virus. They suggest that conclusions based on the absence of the virus, as in the risk assessment for virus-resistant squash, *Cucurbita pepo*, in the USA (Kling 1996) could be critically flawed.

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Transgenic Approaches to Modifying Quality Traits

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ABSTRACT

The present status of transgenic (genetically modified - GM) crops varies greatly between countries. In North America there are large areas of herbicide tolerant and insect resistant GM corn, soybean and cotton, with general consumer acceptance. However, in Europe there has been considerable resistance to foodstuffs produced from GM crops. This diversity of approach has led to confusion and complexity, particularly as regards cross-border trade and the requirements for segregation and/or labelling of GM commodities. It is widely accepted that a large part of the European reluctance derives from a lack of perceived consumer benefits, with the present generation of GM varieties mostly providing agronomic benefits for the growers. This review describes some of the 'second generation' products, specifically those with 'product quality' traits, some of which will provide direct consumer benefits. Many such products are already undergoing field testing in the USA.

INTRODUCTION

To date, most of the GM crop varieties grown around the world contain introduced bacterial genes providing tolerance to broad spectrum herbicides (eg glyphosate or glufosinate) and/or resistance to insect pests such as the European corn borer. Although these varieties now predominate in some areas of North America they are still undergoing small scale testing in Europe where the need for these products and their safety is being questioned by some groups. In the UK this uncertainty has affected the attitude of major retailers who are attempting to guarantee the 'non-GM status' of their products. Such demands have inevitably led to a reassessment of the policy of those US growers who provide commodity crops (principally soybean and maize) to the food processing industry in Europe, and there is consequently much debate about the future success of GM varieties. Most contributors to this debate accept that the lack of direct consumer benefit has been a significant deterrent to acceptance of this technology, particularly against a background of other food scares. Whether self interest will overcome this resistance at some stage depends upon the development of new products where the real and obvious benefit can be seen to be outweigh the assumed and non-obvious risk.

This review will consider some of the ongoing research and development programmes on GM crops, and will concentrate on those potential products with improved quality traits. It will use data assembled from the usual research literature together with information collated from US field trial applications (http://www.nbiap.vt.edu/cfdocs/fieldtests1.cfm) (Table 1) and from various patent databases (http://patents.uspto.gov/access/search-adv.html) (http://pctgazette.wipo.int/) (http://www.patents.ibm.com/). The former database includes information on GM trials of 63 species, with the data subdivided according to the type of introduced gene. In addition, the latter patent sources, which can now consulted on-line at no

cost, are of especial value as they provide a good summary of present commercial priorities, and they include many results that have not yet been published in journal articles.

The various types of research programme will be categorised according to the various objectives which range from industrial objectives, to alterations in seed quality traits and food constituents (Dunwell, 1998), to more long term strategies (Dunwell, 1999) to use crops as a source of valuable medical products. Modifications of agronomic traits (Dunwell, 2000) will not be considered here. In light of the extensive coverage in these three reviews emphasis will be given below only to recent reports.

Table 1. Selected summary of US applications for field trials of genetically modified crops with product quality traits in year 2000 (most recent first).

Application number	Crop	Organisation	Gene - Donor species; Effect (CBI: confidential business information)
00-123-03N	Soybean	Monsanto	Stanol increased
00-119-12N	Corn	Wilson Genetics	Lysine level increased
00-119-10N	Alfalfa	W-L Research	Lignin levels decreased
00-119-04N	Barley	Washington State U	Heat stable glucanase produced
00-112-09N	Corn	n/a	1.) Aspartokinase - Donor: *E. coli* 2.) Dihydrodipicolinate synthase- Donor: Corn; Lysine level increased
00-112-05N	Petunia	Monsanto	CBI - Donor: CBI; Extended flower life
00-110-02N	Rapeseed	Monsanto	CBI - Donor: CBI; Oil profile altered
00-108-15N	Potato	Monsanto	CBI - Donor: CBI; Bruising reduced
00-105-06N	Barley	ARS	Glutenin - Donor: Wheat; Storage protein altered
00-103-05N	Corn	Monsanto	CBI - Donor: CBI; Tryptophan level increased
00-102-03N	Corn	Monsanto	CBI - Donor: CBI; Phytate reduced
00-098-06N	Melon	Agritope	S-adenosylmethione hydolase - Donor: *E. coli;* Fruit ripening altered
00-098-04N	Potato	ARS	Trans-aldolase antisense - Donor: Potato; Blackspot bruise resistant
00-096-06N	Potato	ARS	UDP glucose glucosyltransferase; Steroidal glycoalkaloids reduced
00-094-03N	Tobacco	Ball Helix	Isopentenyl transferase - Donor: *A. thaliana*;

			Leaf senescence delayed
00-094-07N	Corn	Monsanto	1.) CBI - Donor: CBI 2.) Storage protein - Donor:Corn; Methionine level increased
00-082-15N	Tomato	Lipton	CBI - Donor: CBI; Antioxidant enzyme increased
00-080-17N	Corn	Iowa State U	Isoamylase-type starch debranching enzyme - Donor: Corn; Carbohydrate metabolism altered
00-056-16N	Apple	Oregon State U	Sorbitol dehydrogenase - Donor: Apple; Sugar alcohol levels increased
00-054-13N	Rapeseed	Cargill	1.) Acyl-ACP thioesterase - Donor: Rapeseed 2.) Delta-12 saturase - Donor: Rapeseed 3.) Delta-15 desaturase - Donor: Rapeseed 4.) Delta-9 desaturase - Donor: Rapeseed 5.) Delta-9 desaturase - Donor: Soybean 6.) Delta-9 desaturase antisense - Donor: Rapeseed 7.) Fatty acid elongase - Donor: Rapeseed Fatty acid metabolism altered
00-025-01N	Tomato	U of Florida	Ethylene receptor protein antisense - Donor: Tomato; Fruit ripening delayed, altered
00-025-03N	Tomato	U of Florida	Agamous-like gene 8 - Donor: *A. thaliana*; Larger fruit

NON-FOOD APPLICATIONS

Lignin

There have been many programmes concerned with the modification of lignin in crops, especially woody species. The justification comes from the importance of this compound in determining the digestibility of plant material by animals and in the industrial equivalent, namely the production of paper pulp, a process that involves the separation of cellulose from the contaminating lignin. Amongst the enzymes whose levels have been modified are cinnamyl alcohol dehydrogenase (CAD). Reduction of this enzyme in alfalfa using antisense methods led to an increase in digestibility (solubility in sodium hydroxide) (Baucher *et al.*, 1999), a result

associated with a red colouration of the stem. This finding was due to a lower syringyl/guaicyl (S/G) ratio and a lower S+G yield, although the total amount of lignin was unchanged. Recent results on similar plants (Russell *et al.*, 2000) suggest that the incorporation of cinnamylaldehydes into lignin is controlled in the same way as that of cinnamyl alcohols. In related studies on *Populus tremuloides*, transgenic plants showing homologous sense suppression of caffeic acid O-methyltransferase (CAOMT), another enzyme in the lignin pathway, had a mottled or complete red-brown colour in the stem (Tsai *et al.*, 1998), whereas those with a reduced amount of 4-coumarate:coenzyme A ligase exhibited up to 45% less lignin but 15% more cellulose. One of the most comprehensive reports is that of Lapierre *et al.* (1999) who evaluated lignin profiles and pulping performance of 2-year-old transgenic poplar lines with altered expression of CAD or CAOMT. The line with the lowest CAD level had a significantly higher content of free phenolic groups, with an associated improvement in lignin solubilization and fragmentation during kraft pulping. Taken together, these results suggest a potential benefit for lignin-modified trees in the pulp industry.

Biodegradable plastic

One of the recurring claims about GM crops is their potential as a production system for biodegradable plastics, particularly poly (beta-hydroxybutyrate) (PHB) and poly (beta-hydroxyvalerate) (PHV). This project, which was developed as an alternative to an expensive, bacterial fermentation system, has progressed to the extent that PHB amounts as high as 7.7% of fresh seed weight have been reported in oilseed rape (Houmiel *et al.*, 1999). This result was achieved by coordinated expression of three bacterial enzymes, beta-ketothiolase, acetoacyl-CoA reducatase and PHB synthase in the leukoplasts of mature seeds. The more valuable copolymer (PHBV) has been similarly produced in oilseed rape and *A. thaliana* (Slater *et al.*, 1999). Despite these successes the commercial viability of the project remains unproven. The beneficial effects of expressing these plastics within cotton fibre cells (John *et al.*, 1999) has also been claimed.

Ornamental crops

Flowers with altered colour were amongst the first GM products to be commercialised (Mol *et al.*, 1999) and there is a continuing interest in this area (Smith *et al.*, 1999).

FOOD APPLICATIONS

Protein

The improvement of seed quality is at the centre of many plant breeding programmes and there is a wide diversity of approaches to the modification of specific traits (Mazur *et al.*, 1999). (Projects associated with overall increases in seed yield are not considered here.) Amongst the novel means of modifying a quantitative trait, namely grain texture, is one comprising the introduction into wheat of genes encoding puroindoline A and B, two lipid binding proteins associated with grain hardness (Morris & Giroux, 1999). Perhaps better known are the programmes aiming selectively to modify the storage proteins or starches of seed. The commercial interest in these approaches is exemplified by the extensive patent activity and the number of field tests of crops with modified protein traits (Table 1). Some of these projects aim

to alter only a specific amino acid (eg methionine, lysine, trytophan) in order to improve nutritional quality whilst other studies have more general objectives. In this latter class, one of the extensive studies is that in which various soybean glycinin genes (A1aB1b) have been transferred to rice (Takaiwa *et al.*, 2000). Analysis of the transgenic plants produced has shown a 20% increase in protein content in some cases (Momma *et al.*, 1999) and the presence of hybrid protein oligomers that combine glycinin and glutelin subunits (Katsube *et al.*, 1999). In a related study (Kim *et al.*, 1999) of the source of bitterness in glycinin, it has been shown that many of the small bitter peptides (<1000 Da) are composed of uncharged polar amino acids as well as hydrophobic amino acids with a charged residue at either end. Presumably the detailed structural information now available from these 7S and 11S storage proteins (Dunwell *et al.*, 2000) will enable directed elimination of such non-palatable regions of the protein.

Carbohydrate

As well as the projects designed to modify protein quality, there are many concerned specifically with changing the profile of starch produced either in the seed or other storage organ such as the tuber of potatoes. Examples include the use of a beta-amylase gene (Frohberg, 2000a), a rice starch granule-bound protein (Frohberg, 2000b) or a debranching enzyme (Kossmann *et al.*, 1999) to produce modified starches in transgenic plants. It is also claimed (Jacques *et al.*, 1999) that an overall increase in the level of stored carbohydrate can be achieved by use of a glycosyl-transferase that catalyzes the formation of soluble glucans. In a similar study (Kawchuk *et al.*, 1999) designed to improve the storage capacity of potato tubers, the amounts of the two enzymes alpha glucan L-type phosphorylase and alpha glucan H-type phosphorylase have been reduced. The transgenic tubers demonstrate a reduced conversion of starch to sugar during storage, thus prolonging dormancy, reducing the incidence of disease, and increasing the storage life. Production of alternative carbohydrates (Heyer *et al.*, 1999), for example the non-calorific carbohydrate fructan, in place of starch is the subject of several studies including those of Smeekens *et al.* (2000) and Koop *et al.* (2000).

Oil

The most recent (at the time of writing) application for a US field test was that from Monsanto for a trial of soybean with an increased amount of stanol (introduced gene unknown). The significance of this lies in the known beneficial effect of consumption of oil/margarine containing elevated amounts of plant sterols and stanols (Nguyen, 1999). These compounds have proven ability to reduce total and LDL cholesterol by inhibiting cholesterol absorption from the human intestine. The stanols are preferred to sterols since they are virtually unabsorbable; consumption of the esterified form of stanol at the rate of 2-3 g/d reduces LDL cholesterol by 10-15% without side effects. It can be presumed that the field trial in question is designed to produce material for such testing. The general area of modifying plant lipids has been reviewed recently (Broun *et al.*, 1999) and the overall level of interest can be estimated by the large number of field trial line with various oil and lipid compositions. Amongst these are lines of soybean and oilseed rape that contain fatty acids with conjugated double bonds. These non-food products are of value as drying agents in paints, varnishes and inks (Cahoon *et al.*, 1999; 2000).

Other food constituents

In additions to the modifications of proteins, starches and oils there are several studies concerned with minor, though important constituents of food. Perhaps the best known is the production of 'golden rice', a GM rice variety that has increased amounts of vitamin A in the endosperm (Ye *et al.*, 1999), thus the 'golden' epithet. This product, generated with funding from international agricultural aid agencies, should be of great value in alleviating juvenile blindness, a condition induced by vitamin A deficiency and found in many developing countries where rice is the staple crop. Another potential improvement in the nutritional value of rice is that found in GM material expressing the ferritin gene from soybean (Goto *et al.*, 1999). Such products may help to overcome iron deficiency. Other approaches to increasing the absorption of iron from dietary constituents include the use of varieties with low phytate contents. Unfortunately, maize varieties which contain the low phytic acid (*lpa*) mutation have recently been described as 'genetically modified' in reports of feeding trials (Mendoca *et al.*, 1998; Spencer *et al.*, 2000), a description bound to cause confusion in light of the similar beneficial effects of GM varieties expressing microbial phytase genes (Van Ooijen, 2000). These latter varieties have particular value in animal feed; this is also the aim of a project in which a protein-engineered thermostable beta-glucanase has been expressed in the endosperm of transgenic barley (Von Wettstein *et al.*, 2000). It should be noted that the same misleading use of the term 'genetically modified' has been applied recently (Edwards *et al.*, 2000) to high protein soybean varieties produced by conventional breeding.

Other recently described GM food products with possible beneficial health effects include those with increased levels of the antioxidant tocopherol (Dellapenna & Shintani, 2000), and those expressing isoflavone synthase (Feder, 2000; Jung *et al.*, 2000), an enzyme that is part of the phenylpropanoid pathway. Improved sweetness in foods is claimed to result from use of the fructokinase gene (Bennett & Kanayama, 2000) or the sweet protein mabinlin (Sun *et al.*, 2000).

MEDICAL APPLICATIONS

Much attention has been given recently to the potential of using plants as a production system for high value compounds of medical importance. Examples include the expression of antibodies (Fischer *et al.*,1999) that may reduce the growth of bacteria associated with dental caries (Hiatt *et al.*, 2000) or that can be used in the treatment of tumours (Russell, 1999; Stoger *et al.*, 1999). Other related projects include the production of recombinant blood factors (Theisen, 1999; Hooker *et al.*, 2000), human milk proteins (Arakawa *et al.*, 1999), human collagen (Ruggiero *et al.*, 2000) and the human secretory protein somatotrophin (Staub *et al.*, 2000). Recent advances in the use of plants to produce vaccines (Lam *et al.*, 2000) have extended to the expression of a cholera-toxin-B-chain-autoantigen chimeric gene construct of value in the prevention and treatment of autoimmune disease (Arakawa *et al.*, 1999; Langridge & Arakawa, 1999). The potential advantage of using plants as production systems include the reduced risk of mammalian viral contaminants, the ability to scale up at low cost and the low maintenance requirements.

Possibly the most advanced use of plants to produce high value enzymes is the example of transgenic maize that has been used to express recombinant egg white avidin and bacterial glucuronidase. These two products, used in various types of diagnosis, are now marketed by Sigma as products A8706 and G2035, respectively (Hood *et al.*, 1999).

METHODS FOR PRODUCTION OF NOVEL PRODUCTS

Amongst the many possible production systems whereby valuable novel products can be separated from GM plants, two recent claims have been made concerning nectar. In the first (Thornburg, 1999) it is stated that the promoter from a gene encoding 'nectarin', a germin-like protein (Dunwell *et al.*, 2000) highly expressed in the nectaries of a variety of *Nicotiana plumbaginifolia*, can be used to drive the expression of any suitable introduced gene. The product would simply be purified from the collected nectar. A similar approach has been taken recently by Cremers *et al.* (2000) who propose allowing insects to collect the nectar and then purifying the gene product from the honey produced. The regulatory acceptance of this latter approach would seem uncertain in light of the present controversy concerning bees and GM crops.

FUTURE PROSPECTS

The future of GM crops designed with modified quality traits depends to a large extent on the direction of public opinion. If such a product, for example a margarine with improved antioxidants and the capacity to reduce damaging cholesterol, can be developed, then self interest will probably prevail. Otherwise, there is likely to be a lengthy period of continuing opposition from a significant proportion of the population.

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Testing foods derived through biotechnology for potential allergens

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ABSTRACT

The modern methods of biotechnology allow the transfer of genes, from any source, into our major food crops. The likelihood of any introduced gene product being an allergen is probabilistically extremely low, but developers of biotechnology crops must specifically address these risks as a part of the safety evaluation process. Definitive immunodiagnostic methods are in place to detect the transfer of known protein allergens, or an increase in their abundance. A combination of genetic and physicochemical criteria can provide reasonable assurance that proteins from sources with no allergenic history pose no significant allergenic concern. Using the decision tree approach developed by the Allergy and Immunology Institute and the International Food Biotechnology Council, that combines diagnostic and predictive criteria, it is possible to assess the potential allergenicity of foods derived through biotechnology. Consistent application of this assessment procedure can provide reasonable assurance that genetically modified foods introduced into the marketplace are as safe as foods derived from new plant varieties developed through traditional breeding.

INTRODUCTION

It is estimated that <1% - 2% of the adult population suffers from food allergies; defined for the purposes of this discussion as type I IgE mediated immunologic reactions to specific foods. The IgE antibody is the least prevalent of the five antibody types, but it is central to the induction of immediate hypersensitivity. Food allergic individuals produce high levels of IgE in response to repeated exposure to particular antigens, whereas normal individuals produce other Ig isotypes and only small amounts of IgE. The IgE molecules bind to high affinity receptors on mast cells and circulating basophils causing them to become sensitised. Cross-linking of the specific antigen to these cell-bound IgE molecules initiates a series of biological responses, including the secretion of histamine, resulting in an allergic inflammatory response.

The general consensus is that the most common allergenic foods, world-wide, are crustacea, egg, fish, milk, peanuts, soybeans, tree nuts and wheat. However, the prevalence of food allergens varies in different parts of the world, according to dietary preferences (for example, Japan has a high reported incidence of rice and buckwheat allergies). In the US, these eight foods account for over 90% of food allergies amongst hypersensitive (atopic) individuals. Allergies to foods such as milk and eggs are most prevalent amongst children and often disappear by adulthood.

In many cases, food allergies are an inconvenience resulting in unpleasant reactions, such as tingling of the lips and mouth or diarrhoea. However, for some individuals, who are highly sensitive to particular foods, such as peanut, the results of consuming even tiny

quantities of that food can be life threatening. These individuals exhibit severe anaphylactic reactions such as bronchospasm, choking, nausea, vomiting and hypotension and typically take rigorous precautions to avoid consumption of foods to which they are sensitive. Appropriate labeling of processed foods containing common food allergens is critical in helping allergic consumers make their dietary selections.

The modern methods of biotechnology allow the transfer of genes, from any source, into our major food crops. This is a cause of concern to food allergic consumers, and places responsibility on developers of new crop varieties to take all reasonable precautions to prevent the transfer of food allergens. To this end, almost all regulations and guidelines directed at novel foods derived through recombinant DNA technology (e.g. US, Food and Drug Administration, 1992) require the developer to address the risk of allergenicity.

The following discussion focuses on addressing concerns about IgE mediated food allergies associated with food crop plants. It does not address gluten sensitive enteropathy (celiac disease), a distinct clinical pathologic entity that is observed in specific individuals sensitive to gluten in certain foods. Nor does not discuss food intolerances, which are generally not well characterised.

DISCUSSION

Almost all food allergens are proteins, but not all proteins are food allergens. The crops from which our staple foods are derived contain tens of thousands of different proteins. The distribution of those proteins varies markedly in different parts of the plant and can be profoundly influenced by environmental factors such as climate and disease pressure. A single plant species may contain as many as 50,000 different proteins.

Despite the huge number and variation of proteins in our diet, it is apparent that very few are food allergens. Moreover, many allergenic proteins share certain properties, for example, they tend to be resistant to degradation in the human gut, are resistant to the conditions to which they are exposed during food processing and have a molecular weight of between 10 and 70 kiloDaltons. Many allergenic proteins are also relatively abundant constituents of food.

Many allergens have been cloned and characterised and their nucleic acid sequences determined. Several years ago, the Allergy and Immunology Institute (AII) of the International Life Sciences Institute and an industry group known as the International Food Biotechnology Council (IFBC) published a report entitled; "Assessment of the Allergenic Potential of Foods Derived from Genetically Engineered Crop Plants." (Metcalf *et al.*, 1996). As well as the eight commonly allergenic food groups that have been widely studied, the report listed more than 160 foods and food related substances that have been associated with allergic reactions in individuals. This list includes most of our major grain, oilseed and vegetable crops as well as processed products such as beer and chocolate. Reports of allergy associated with these less commonly allergenic foods usually involve a very small number of cases. Only occasionally has the association of a particular food with allergic symptoms been confirmed through double blind placebo controlled food challenges (Bock *et al.*, 1988) - the gold standard of food allergy diagnosis.

The report also proposed an approach for assessing the allergenic potential of foods derived from genetically engineered crop plants. It is based on a decision tree strategy that takes account of the source from which a gene was obtained, amino acid sequence comparisons with known allergens, *in vitro* and *in vivo* immunologic analyses as well as an assessment of physicochemical characteristics of the gene product. The decision tree provides guidance to developers in addressing food allergy risks. However, it is important to emphasise that it is the totality of these assessments that provides reasonable assurance that foods derived from new plant varieties will not introduce allergenic concerns beyond those that already exist relative to our current food supply.

The assessment scheme is based on certain principals of allergy assessment.

- The transfer of known allergens into foods should be avoided.
- As a precautionary principal, developers should assume that any gene from a known allergenic source encodes an allergen until proven otherwise.
- The allergenic potential of each introduced gene should be assessed and, if it is determined that an allergen has been transferred, then consumers must be informed by appropriate labeling of foods containing the gene product.

The first consideration is the source of the introduced gene and whether the gene is from a commonly allergenic food, a less commonly allergenic food or other known allergen or, a source with no allergenic history.

As a preliminary step in assessing allergenic concerns, the amino acid sequence of genes from all sources is compared against a database of all known allergens, screening for immunologically significant sequence similarities. Such publicly available databases can be accessed through the internet and include:

- GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html),
- EMBL (http://www.embl-heidelberg.de/Services/index.html),
- SwissProt (http://cbrg.inf.ethz.ch/section3_1.html).

To facilitate that analysis, the AII/IFBC report referenced 198 sequences of food and non-food proteins that are reported to be allergens, and which can be accessed from these databases. This list continues to grow as additional allergens are characterised.

The sequence homology is examined for structural relationships using a computer program such as FASTA (Pearson & Lipman, 1988) looking for theoretical epitope matches (an epitope is a specific amino acid sequence on the surface of an antigen to which an antibody binds). Based on a generalised minimal peptide length for T-cell binding epitopes, any sequence identity comprising eight contiguous amino acids is viewed as an indicator of potential allergenicity and requires the gene product undergo further testing. There are limitations of this analysis, especially as it relates to discontinuous epitopes (i.e., where the antigen binding site is formed from two or more non-contiguous sequences of amino acids brought into close proximity by folding of the amino acid chain). It has also been suggested that homologies of fewer than eight contiguous amino acids should be

considered significant. However, at that level of resolution, many proteins with no history of food allergy show significant homologies.

If proteins encoded by genes derived from sources with no history of allergy show significant homologies, they should be evaluated using an approach designed for genes derived from less-commonly allergenic foods.

Testing of transgenic crops containing novel proteins expressed by genes from commonly allergenic foods is relatively straightforward. Sera from individuals sensitive to the food are usually available and the protein can be tested in a series of *in vitro* solid phase immunoassays, for example, a radioallergosorbent test (RAST™) or RAST™ inhibition assay (Adolphson *et al.*, 1986) or an enzyme linked immunosorbent assay (ELISA). A positive reaction in an *in vitro* test raises concerns that the novel protein might be a food allergen. Unless this possibility can be convincingly discounted by additional *in vivo* testing, food containing the newly introduced protein should be clearly labeled as to the source of the gene before being placed on the market. As a practical matter, it is unlikely that any developer would decide to proceed to market if foods derived from the new crop variety must be labeled as containing a food allergen.

In the case of negative or equivocal results with the solid phase immunoassays, the food should be investigated further using *in vivo* skin prick tests (Norman, 1986) with an appropriate number of sensitive test subjects. In view of the potential risk to the subject, skin prick tests should only be conducted with the approval of an Institutional Review Board (IRB). A positive skin prick test raises the same concerns as a positive *in vitro* reaction requiring foods containing the newly introduced gene to be labeled.

Foods containing new gene products derived from commonly allergenic foods that fail to cause positive reactions in solid phase immunoassays or skin prick tests are unlikely to contain allergens. Nevertheless, the AII and IFBC recommends that the absence of allergens from commonly allergenic foods should be confirmed by a double blind placebo controlled feeding challenge (Bock *et al.*, 1988) using an appropriate number of sensitive subjects. A double blind placebo controlled feeding challenge should only be conducted with the approval of an IRB. Foods that fail to elicit a reaction in such a challenge are very unlikely to contain an allergen from a commonly allergenic food and need not be labeled as to the source of the newly introduced gene.

Most allergenic foods contain multiple allergenic components, often classified as major and minor allergens. A major allergen is one to which >50% of individuals sensitive to that food react; minor allergens elicit a response in a smaller percentage of sensitive individuals. The great majority of food allergic individuals are sensitive to one or more of the major allergens present in commonly allergenic foods. Each food should be tested against immune sera from a minimum of 14 sensitive individuals with documented histories of sensitivity to the corresponding food (i.e. verified sera). Similarly, a double blind placebo controlled feeding challenge should be conducted with a minimum of 14 sensitive subjects. This will ensure a >99.9% probability of detecting the presence of a major food allergen and >95 % probability of detecting a minor allergen to which >20% of the sensitive

population reacts. If only 5 sera are available, there is still a 95% probability of detecting a major allergen.

It is extremely difficult to find individuals who are sensitive to many of the less commonly allergenic foods because these conditions are often very rare. While the aim should still be to obtain sera from 14 individuals, the AII and IFBC consensus was that gene products from less commonly allergenic foods that fail to react with five or more sera should still be available for use in food products without labeling. If less than five sera are tested (i.e., there is less than 95% probability of detecting the presence of a major allergen) and they yield negative results, then the gene product should be subjected to physicochemical evaluation. This should include digestibility in an *in vitro* gastric model and immunochemical stability after being subjected to physical conditions typical of processing for that food. Gene products that are sensitive to digestion or processing should be available for use in food products without labeling. Otherwise, developers should discuss the results with the appropriate regulatory authorities to determine how to proceed. A factor to be considered in those discussions may be the concentration of the gene product in foods since many major food allergens are present in high concentrations in food (e.g., glycinin in soybean, ovalbumin in egg and casein in milk).

These attributes are also considered important in predicting the potential allergenicity of gene products derived from less commonly allergenic foods or foods with no history of consumption.

Gene products that prove resistant to digestion and/or heat denaturation may have a greater potential to be food allergens. Such resistant proteins have a greater chance of surviving intact to cross the intestinal mucosa and stimulate an allergenic reaction. The digestibility of proteins is evaluated in simple test-tube models that simulate the conditions of digestion in the human stomach and intestines (United States Pharmacopeial Convention, Inc., 1990). Thermal stability is investigated under temperatures and pressures equivalent to those encountered during food processing. However, it should be emphasised that not all indigestible and heat stable proteins are food allergens, for example, horse radish peroxidase and glutamate decarboxylase have been reported to be stable in simulated gastric fluid, but have no history of food allergy.

Many allergens are major components of foods (e.g., storage proteins that may comprise >25% of a seed). Total dietary exposure, coupled with age at the time of exposure, seem to be factors in sensitisation and should be considered. Properties such as molecular weight and glycosylation are not considered reliable predictors of allergenicity.

Assessment of gene products from sources with no allergenic history begins with a comparison of the amino acid sequence against the database of all known allergens, screening for immunologically significant sequence similarities. Foods containing gene products that show amino acid similarity to known allergens, are assessed in the same manner as foods containing a gene from a less commonly allergenic food or other known allergens.

Gene products from sources with no allergenic history that lack immunologically significant sequence identity to known allergens should still be subjected to physicochemical evaluation before concluding that they are unlikely to be food allergens and do not require labeling. Developers should consult with the appropriate regulatory agency in cases where such gene products lack immunologically significant sequence identity to known allergens, but show significant resistance to digestion and/or processing. The EPA recently held a Science Advisory Panel to consider the food safety of Cry9C, a protein isolated from *Bacillus thuringiensis* and introduced into maize plants to derive tolerance to European corn borer (*Ostrinia nubilalis*) (http://www.epa.gov/oppbppd1/biopesticides/cry9c/cry9c-peer_review.htm). The Cry9 protein shows no homology to known allergens, but is reportedly indigestible and heat resistant. The outcome of the panel's deliberations is unknown at the time of writing.

There is considerable interest in the potential of animal models to predict the allergenic potential of gene products. Work is being conducted on several candidate models, including the brown Norway rat, C3H/HeJ mouse, guinea pig and dog. The AII and IFBC carefully considered the use of these animal models to evaluate the potential allergenicity of gene products. They agreed that models can be useful to investigate specific mechanisms of food allergy, but concluded that, at the present time, there are no animal models that are reliably predictive of the allergenic potential of foods for humans. Animals sensitised to particular foods could prove useful in confirming the absence of any unintended increases in endogenous levels of allergens.

An example of how companies apply the decision tree is provided by a research project aimed at improving the amino acid composition of soybean meal through biotechnology. Soybeans are deficient in the essential amino acid methionine, which must be added separately to animal feed. Brazil nuts are known to be unusually high in methionine and researchers were able to isolate and transfer a high methionine seed storage gene (2S albumin) from Brazil nut into soybean with the result that the soybeans now contained much higher levels of methionine. Brazil nuts are known to be a food allergen for a small group of people. The company, therefore, set out to confirm that the transferred gene did not encode a food allergen before proceeding to commercial development of a high methionine soybean line, in compliance with the FDA guidelines.

After an extensive search, lasting nearly two years, nine different sera were obtained from patients with documented histories of sensitivity to Brazil nuts. The pooled sera were tested against extracts from the transgenic high methionine beans and gave a positive result in a RAST™ inhibition assay (Nordlee *et al.*, 1996). Eight of the nine sera were shown, by Western blotting, to contain IgE antibodies that bound to the Brazil nut 2S albumin (IgE antibodies in the ninth serum bound to a different Brazil nut protein). The IgE antibodies in these eight sera also bound to a protein in the transgenic soybeans of the same size as the Brazil nut 2S albumin that was absent from non-transgenic soybeans. Three Brazil nut sensitive volunteers were skin prick tested with extracts from Brazil nuts and the high methionine beans. All three reacted to both extracts at dilutions ranging from 1:1,000,000 to 1:1,000, but not to extracts from normal soybeans.

In the light of these findings, the company terminated the project. None of the transgenic beans ever entered the food supply. This experience clearly shows companies developing new crop varieties are aware of the need to address the potential risks of allergenicity.

The AII and IFBC focused on the potential to move allergens from one food to another. However, developers are also concerned that genetic manipulations do not significantly increase the abundance of allergenic proteins and perhaps increase the risk of inducing food allergy. Western blotting can be used to investigate the distribution of allergenic proteins in the edible parts of crops, especially those such as soybean or wheat, that contain multiple allergens, and to determine if genetic modification has inadvertently caused a significant increase in the abundance of any of those allergens. Soybeans have been genetically modified to increase their oleic acid content in order to improve the nutritional qualities and heat stability of soybean oil. The high oleic acid soybeans were tested against sera from six individuals with a documented history of soybean allergy who had IgE antibodies to a variety of different soybean proteins. Western blots of the proteins from transgenic high oleic soybean and non-transgenic soybean showed no quantitative or qualitative differences in IgE binding. On this basis, the company concluded that the genetic modification had not increased the allergenic potential of the high oleic soybeans (DuPont Agricultural Products, 1997).

In summary, the likelihood of an introduced protein being an allergen is probabilistically extremely low. Unlike the majority of food allergens, most proteins introduced into crops are unstable in the human gut and are easily destroyed by processing. Moreover, only minute amounts of a protein are usually required to achieve the desired modification. Definitive immunodiagnostic methods are in place to detect the transfer of known allergens, or an increase in their abundance. A combination of genetic and physicochemical criteria provides reasonable assurance that proteins from sources with no allergenic history pose no significant allergenic concern. Using the decision tree approach developed by the AII and IFBC, that combines diagnostic and predictive criteria, it is possible to assess the potential allergenicity of genetically modified foods. Consistent application of this assessment procedure can provide reasonable assurance that genetically modified foods that are introduced into the marketplace are as safe as foods derived from new plant varieties developed through traditional breeding.

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Switchable transgenes

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