
Technologies for Biological Containment of GM and Non-GM Crops

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Glossary

allele	alternative form of a gene
angiosperm	flowering plants with enclosed seeds
anther	pollen sac of the stamen of angiosperms, in which pollen grains develop
APHIS	Animal & Plant Health Inspection Service (United States)
apomixis	the production of seed in the absence of sexual fertilisation
ARS	Agricultural Research Service, of the USDA
backcrossing	crossing of hybrids to one of its parents
BBC	British Broadcasting Corporation
Bt	<i>Bacillus thuringiensis</i>
cecropin	basic polypeptides with antibacterial activity
chloroplast	organelle of plant cells
cleistogamy	self fertilisation within a closed flower
clones	individual of the same genetic constitution as its progenitor
CSIRO	Commonwealth Scientific and Industrial Research Organisation, of Australia
cytoplasm	entire components of a cell, excluding the nucleus
cytotoxic	causing cell death
DEFRA	Department of the Environment, Food and Rural Affairs (United Kingdom)
DNA	di-deoxy nucleic acid
E	east
EC	European Community
egg cells	the female gamete containing a haploid nucleus, located in the ovary
embryo	the structure that develops from a zygote following fertilisation, up to the time of germination
ES	Spain
EU	European Union
EU25	European Union, 25 member states

F₁	the first hybrid offspring following cross-fertilisation
FAO	Food & Agriculture Organisation of the United Nations
feral	having escaped cultivation or domestication and reverted to a wild state
fertilisation	fusion of male and female gametes to form a zygote
flora	collective term for all plants characteristic of a place or period
FR	France
gene	functional unity of heredity; DNA sequence that encodes polypeptides
gene flow	the loss or gain of alleles from a population due to the movement of fertile individuals or the transfer of gametes
genome	the complete compliment of an organism's genes
GFP	green fluorescent protein
glufosinate	a systemically acting, non-selective herbicide, present in Basta, Rely, Finale, Challenge and Liberty Link
glycoprotein	any protein containing covalently bound carbohydrate
glyphosate	a systemically acting, non-selective herbicide, present in Roundup and Tumbleweed
GM	genetically modified
GMO	genetically modified organism
GURT	genetic use restriction technology
guttation fluid	exudates from leaves and roots in plants
gymnosperm	vascular plants bearing naked seed not enclosed in any specialised chambers
gynoecium	the female organ of plants comprising stigma, style and ovary
ha	hectares
heterozygous	having two different alleles for a given gene or genetic character
homozygous	having two identical alleles for a given gene or genetic character
HT	herbicide tolerant
<i>in planta</i>	within the plant, as grown in ordinary conditions
<i>in vitro</i>	grown under aseptic conditions
intein	internal peptide sequence of an protein precursor that is spliced out by transpeptidation during processing to form a mature protein
introgression	the transplantation of genes between species as a result of cross-pollination
ISB	Information Systems for Biotechnology (Virginia Tech)
KTRDC	Kentucky Tobacco Research & Development Center
LSBC	Large Scale Biology Corporation

magainin	antibiotic peptides from <i>Xenopus</i> that act against bacterial membranes
mycorrhizae	mutualistic association of plant roots and fungi
N	north
NEPA	National Environmental Policy Act; the environmental policy of the US Government
nucleus	the chromosome containing organelle of a eukaryotic cell
OECD	Organisation for Economic Co-operation and Development
out-crossing	the crossing of two individuals to form a hybrid
perennial	a plant that lives for several years
phytoremediation	the use of plants to reduce levels of pollutants
plastid	plant organelles including chloroplasts, chromoplasts and amyloplasts
pollen	the male gametophyte of plants that develops within the anthers of stamens
pollen cells	individual male gametophytes of plants
pollination	the placement of pollen on to the stigma of flowers as a precursor to fertilisation
protease	an enzyme that hydrolyses one or more peptide bonds in a protein, leading to degradation and non-function
proteins	a biological polymer consisting of amino acids, encoded by DNA sequences
PT	Portugal
recombinant	resulting from several different origins; recombinant proteins are encoding within cells from DNA sequences that have been inserted from another organism
recombinase	an enzyme that recognises specific sites in a nucleotide sequence and binds sites on different DNA molecules
rhizomes	underground stem of a plant, often horizontal, non-green and root-like in appearance
ribonuclease	a group of enzymes that cleave phosphodiester bonds of RNA and leading to degradation and non-function
RNAi	RNA interference
Roundup	a systemic non-selective herbicide
S	south
self-compatible	capable of the generation of fertile offspring from fertilisation of pollen and egg cells of the same individual plant
sp.	species
sperm cells	the male gamete, enclosed in pollen cells in plants

splicing	the linkage of two strands of duplex DNA at complementary single-stranded terminations by means of DNA ligase
spp.	species (plural)
sq Km	square kilometers
ssp.	sub-species
stolons	horizontally growing underground stem that forms a new plant at the end
symbiotic	ecological relationship between two different species that co-exist in direct contact
terminator	term adopted to describe a genetic containment system that generates sterile seeds
TM	transgene mitigation
TMV	tobacco mosaic virus
transgene	a functioning gene inserted inserted into another organism to modify its performance
UK	United Kingdom
US	United States
USDA	United States Department of Agriculture
vector	organism that carries an organism or gene to another species host
W	west
WHO	World Health Organisation
zygote	the diploid product following the union of haploid gametes; a fertilised egg

Executive Summary

International Perspective

The development of GM technology continues to expand into increasing numbers of crops and conferred traits. Inevitably, the focus remains on the major field crops of soybean, maize, cotton, oilseed rape and potato with introduced genes conferring herbicide tolerance and/or pest resistance. Although there are comparatively few GM crops that have been commercialised to date, GM versions of 172 plant species have been grown in field trials in 31 countries.

European Crops with Containment Issues

Of the 20 main crops in the EU there are four for which GM varieties are commercially available (cotton, maize for animal feed and forage, and oilseed rape). Fourteen have GM varieties in field trials (bread wheat, barley, durum wheat, sunflower, oats, potatoes, sugar beet, grapes, alfalfa, olives, field peas, clover, apples, rice) and two have GM varieties still in development (rye, triticale). Many of these crops have hybridisation potential with wild and weedy relatives in the European flora (bread wheat, barley, oilseed rape, durum wheat, oats, sugar beet and grapes), with escapes (sunflower); and all have potential to cross-pollinate fields non-GM crops. Several fodder crops, forestry trees, grasses and ornamentals have varieties in field trials and these too may hybridise with wild relatives in the European flora (alfalfa, clover, lupin, silver birch, sweet chestnut, Norway spruce, Scots pine, poplar, elm, *Agrostis canina*, *A. stolonifera*, *Festuca arundinacea*, *Lolium perenne*, *L. multiflorum*, statice and rose). All these crops will require containment strategies to be in place if it is deemed necessary to prevent transgene movement to wild relatives and non-GM crops.

Current Containment Strategies

A wide variety of GM containment strategies are currently under development, with a particular focus on crops expressing pharmaceutical products. Physical containment in greenhouses and growth rooms is suitable for some crops (tomatoes, lettuce) and for research purposes. Aquatic bioreactors of some non-crop species (algae, moss, and duckweed) expressing pharmaceutical products have been adopted by some biotechnology companies. There are obvious limitations of the scale of physical containment strategies, addressed in part by the development of large underground facilities in the US and Canada. The additional resources required to grow plants underground incurs high costs that in the long term may negate any advantage of GM for commercial production.

Natural genetic containment has been adopted by some companies through the selection of either non-food/feed crops (algae, moss, duckweed) as bio-pharming platforms or organisms with no wild relatives present in the local flora (safflower in the Americas). The expression of pharmaceutical products in leafy crops (tobacco, alfalfa, lettuce, spinach) enables growth and harvesting prior to and in the absence of flowering.

Transgenically controlled containment strategies range in their approach and degree of development. Plastid transformation is relatively well developed but is not suited to all traits or crops and does not offer complete containment. Male sterility is well developed across a range of plants but has limitations in its application for fruit/seed bearing crops. It has been adopted in some commercial lines of oilseed rape despite not preventing escape via seed. Conditional lethality can be used to prevent flowering or seed development following the application of a chemical inducer, but requires 100% induction of the trait and sufficient application of the inducer to all plants. Equally, inducible expression of the GM trait requires equally stringent application conditions. Such a method will contain the trait but will allow the escape of a non-functioning transgene. Seed lethality ('terminator' technology) is the only strategy at present that prevents transgene movement via seed, but due to public opinion against the concept it has never been trialled in the field and is no longer under commercial development.

Methods to control flowering and fruit development such as apomixis and cleistogamy will prevent crop-to-wild and wild-to-crop pollination, but in nature both of these strategies are complex and leaky. None of the genes controlling these traits have as yet been identified or characterised and therefore have not been transgenically introduced into crop species. Neither of these strategies will prevent transgene escape via seed and any feral apomicts that form are arguably more likely to become invasives.

Transgene mitigation reduces the fitness of initial hybrids and so prevents stable introgression of transgenes into wild populations. However, it does not prevent initial formation of hybrids or spread to non-GM crops. Such strategies could be detrimental to wild populations and have not yet been demonstrated in the field. Similarly, auxotrophy prevents persistence of escapes and hybrids containing the transgene in an uncontrolled environment, but does not prevent transgene movement from the crop.

Recoverable block of function, intein *trans*-splicing and transgene excision all use recombinases to modify the transgene *in planta* either to induce expression or to prevent it. All require optimal conditions and 100% accuracy to function and none have been tested under field conditions as yet. All will contain the GM trait but all will allow some non-native DNA to escape to wild populations or to non-GM crops.

There are particular issues with GM trees and grasses as both are largely undomesticated, wind pollinated and perennial, thus providing many opportunities for hybridisation. Some species of both trees and grass are also capable of vegetative propagation without sexual reproduction. There are additional concerns regarding the weedy nature of many grass species and the long-term stability of GM traits across the life span of trees. Transgene stability and conferred sterility are difficult to trial in trees as most field trials are only conducted during the juvenile phase of tree growth.

Bio-pharming of pharmaceutical and industrial compounds in plants

Bio-pharming of pharmaceutical and industrial compounds in plants offers an attractive alternative to mammalian-based pharmaceutical and vaccine production. Several plant-based products are already on the market (Prodigene's avidin, β -glucuronidase, trypsin generated in GM maize; Ventria's lactoferrin generated in GM rice). Numerous products are in clinical trials (collagen, antibodies against tooth decay and non-Hodgkin's lymphoma from tobacco; human gastric lipase, therapeutic enzymes, dietary supplements from maize; Hepatitis B and Norwalk virus vaccines from potato; rabies vaccines from spinach; dietary supplements from *Arabidopsis*). The initial production platforms for plant-based pharmaceuticals were selected from conventional crops, largely because an established knowledge base already existed. Tobacco and other leafy crops such as alfalfa, lettuce and spinach are widely used as leaves can be harvested and no flowering is required. Many of these crops can be grown in contained greenhouses. Potato is also widely used and can also be grown in contained conditions. The introduction of morphological markers may aid in the recognition and traceability of crops expressing pharmaceutical products.

Plant cells or plant parts may be transformed and maintained in culture to produce recombinant products in a contained environment. Plant cells in suspension or *in vitro*, roots, root cells and guttation fluid from leaves may be engineered to secrete proteins that may be harvested in a continuous, non-destructive manner. Most strategies in this category remain developmental and have not been commercially adopted at present.

Transient expression produces GM compounds from non-GM plants via the utilisation of bacterial or viral vectors. These vectors introduce the trait into specific tissues of whole plants or plant parts, but do not insert them into the heritable genome. There are some limitations of scale and the field release of such crops will require the regulation of the vector. However, several companies have several transiently expressed products in clinical and pre-clinical trials from crops raised in physical containment.

1. Introduction

Genetically modified (GM) crops were first grown commercially on a small scale in 1995 but have since consistently increased the global area under cultivation. Specifically, between 1996 and 2004 the amount of land assigned to GM crops has expanded by more than 47-fold, from 1.7 Mha to 81 Mha, whilst the number of countries involved has similarly increased from 4 in 1996 to 17 in 2004 (James 2005). Consumer and political resistance to the technology mean that this trend has not been followed in Europe (Dunwell 2005; Tencalla 2005). For this reason, the global acreage of GM crops is highly skewed, with USA (Runge & Ryan 2004), Canada, Argentina, China, Brazil and South Africa collectively accounting for >99% of all GM crops grown. Whilst there has been a *de facto* moratorium on the cultivation of GM crops within the UK since 1999, elsewhere in Europe, there has been limited commercialisation of GM Bt corn and GM HT soybean, particularly in Spain (Table 1.1).

Table 1.1 Transgenic Maize Varieties Inscribed in the Commercial Varieties' Register of Spain.
(Data obtained from USDA 2005)

Event	Strain	Company	Date
Bt-176	Compa CB	Syngenta	26 March 1998
(SYN-EV176-9)	Jordi CB		26 March 1998
	Brama		11 March 2003
	Escobar		16 February 2004
MON-810	Alican BT	Limagrain	11 March 2003
(MON-00810-6)	Aristis BT	Nickson Sur	11 March 2003
	DKC 6575	Monsanto	11 March 2003
	PR33P67	Pioneer Hi-Bred	11 March 2003
	Campero Bt	Advanta	16 February 2004
	Cuartal Bt	Arlesa	16 February 2004
	DKC 6550	Monsanto	16 February 2004
	Bambier Bt	Nickson Sur	16 February 2004
	Jaral Bt	Semillas Fitó	16 February 2004
	PR 32 P76	Pioneer Hi-Bred	16 February 2004
	Portect	Koipesol	16 February 2004
	Elgina, Olimpica	Pioneer Hi-Bred	EU catalogue
	Bolsa, Levina	Pioneer Hi-Bred	17 September 2004
	Novelis	Coop de Pau	17 September 2004
	DK 513	Monsanto	17 September 2004

1.1 Potential benefits of GM crops

GM crops offer a range of potential benefits that may be environmentally advantageous. There is a global need to move towards the sustainable generation of the food, feed, fuel and fibre needs of an ever expanding population on limited and degrading land resources (Nickson 2005). The global population has more than doubled in the last 50 years from 2.5 billion in 1950 to over 6 billion at present. Population pressure is reducing land and water resources by development, industry and inappropriate agricultural practices. There are also insecurities regarding the future of agriculture in climatically marginal areas due to the uncertainties of climate change. In addition, there are pressures on maintaining areas for conservation and to sequester carbon from the atmosphere. The development of sustainable agriculture in the face of such pressure on resources is a challenge for this and future generations.

The adoption of GM technology has the potential to reduce the inputs required in agricultural systems (Bennett *et al.* 2004a,b). Crops engineered with resistance to insects (Bates *et al.* 2005) and to herbicides require substantially simplified management practices compared to conventional varieties and reduction in losses leading to increased yields (Pray *et al.* 2002; Nickson 2005). Crops that require less in-the-field management result in lower on-farm fuel consumption. In addition, fields require less tillage between crops to manage weeds and as a result, no-tillage and conservation tillage practices may reduce soil erosion (Fawcett & Towery 2003; Nickson 2005). The cultivation of crops expressing insect resistance has resulted in a reduction of pesticide applications on crops and thereby reduced operator exposure (Hosseini *et al.* 2004; Huang *et al.* 2005). This results in fewer residues in food and feed crops, less chemical release into the environment and a potential increase in on-farm diversity in insects and pollinators (Nickson 2005). Thus there is a subsequent increase in efficiency and a potential reduction in the negative environmental footprint of agriculture.

The next generation of GM crops (Dunwell 2005) will have increased resistance to fungal and viral diseases and will result in further efficiency advantages compared to non-GM. The introduction of genes conferring tolerance to abiotic stress (Suzuki *et al.* 2005) such as drought or inundation, extremes of heat or cold, salinity, aluminium and heavy metals will enable marginal land to become more productive and may facilitate the remediation of polluted soils (Czako *et al.* 2005; Uchida *et al.* 2005). This would allow an increase in the productive land area without any loss of natural habitat. As the number of crops carrying such traits increases, growers have a greater choice of crops enabling better management of production and management against risk, and it is claimed that this will make communities better placed to cope with environmental problems such as drought (Nickson 2005).

Future GM crop development is also investigating improved output traits such as product quality and value (Dunwell 2005). Improvements in dietary and nutritional value, storage and shelf life and removal of allergens from crops such as peanuts (Dodo *et al.* 2005), soybean (Herman *et al.* 2003) and oilseed rape are being developed and some are being trialled. GM technology also offers potential improvements to compositional traits of starch, proteins and oils, sugar quality, the baking quality of bread wheat and malting quality of barley. Improvements to the nutritional value of fodder and feed crops will increase the efficiency of livestock production systems (Nickson 2005). In addition, the use of GM crops as production platforms for industrial chemicals and pharmaceuticals (see Section 4 below) will reduce reliance on the chemical industry and on animal-based production systems. Such improvements in output quality should all be possible without increasing inputs with the adoption of GM crops.

The potential environmental impact of not adopting GM technology is considered by some to be greater than the environmental risks currently posed by GM crops themselves (Nuffield 2003; Nickson 2005).

1.2 Potential risks of GM crops

Concerns regarding the safety of GM crops focus on three issues of food safety, agronomic safety and environmental safety. These issues have been extensively reviewed and investigated (Snow & Moran-Palma 1997; Kaeppler 2000; Shelton *et al.* 2000; Wolfenbarger & Phifer 2000; Dale *et al.* 2002; GM Science Review Panel 2003, 2004; Kok & Kuiper 2003; Martinez-Ghersa *et al.* 2003; Tabashnik *et al.* 2003; Thomson 2003; Snow *et al.* 2005). Food safety concerns relate to the potential immunogenicity and allergenicity (Goodman *et al.* 2005; Lehrer & Bannon 2005; Prescott *et al.* 2005) and possible reduced quality of food products expressing genes that are resistant to insect, fungal and viral pathogens or tolerant to herbicide application. Agronomic safety issues relate to the potential impact on the agricultural environment of the transfer of pest resistant and herbicide tolerance traits to weedy species, and of the persistence of feral crop plants carrying these traits. Environmental safety issues focus on the direct or indirect effects of GM crops on non-target organisms and the transfer of GM traits to populations of wild plants (FAO 2003; Pilson & Prendeville 2004).

The transfer of GM traits from crops to wild relatives and non-GM crops occur by the same routes. Gene flow via pollen to a wild or cultivated plant may lead to the formation of F₁ hybrids expressing the GM trait; or seed escape during harvest, transportation or processing that may enable the establishment of feral crop populations expressing the GM trait. Any feral plants that persist may facilitate further gene flow between crop plants and wild relatives (Claessen *et al.* 2005).

The impact of a particular transgene in the wild is dependent on several factors. If the GM trait confers a selective advantage over wild plants, then persistence and introgression of this trait into wild or weedy populations is more likely (Jenczewski *et al.* 2003). If the trait confers a physiological disadvantage, then selection pressure is against the trait and individuals containing the transgene will be competed out of the population. For these reasons, different GM traits have the potential to cause different environmental and agronomic impacts.

Traits such as inherent resistance to insect, fungal and viral infection will undoubtedly confer an advantage over plants lacking these traits and this increased fitness may lead to an increase in transgene frequency in the wild. This may have further downstream effects in terms of the dynamics of insect populations and the organisms that predate them. The extent of the benefit will depend upon the severity of the infection (Pilson & Prendeville 2004). Movement of herbicide tolerance traits into wild populations will only confer an advantage where herbicides are applied. The physiological effort of sustaining the trait in the absence of herbicide selection may be costly in the longer term (Snow *et al.* 1999; Gueritane *et al.* 2002) resulting in selection against these plants. The processes of genetic drift in the population will determine the fate of traits that confer no benefit (Pilson & Prendeville 2004).

The movement of GM traits conferring selective advantage into wild plant populations has the potential to reduce the number or diversity of wild plants and so alter the ecological structure of communities. Wild relatives may in effect become extinct as a result of swamping by competitive plants and repeated hybridisation. Specific traits such as drought and salt tolerance may allow plants carrying these transgenes to invade new habitats and out-compete native plants leading to unwanted ecological change.

1.3 Risk assessment principles and gene flow

The future of GM crops in Europe remains uncertain (Lheureux *et al.* 2003). It depends to some extent on a change in public attitudes towards the perceived risks presented by the technology but largely on the regulators' evaluation of the actual risks involved (Anon 2002b). As with other aspects of life, perception of risk need not bear any relationship to the actual risks involved. Nevertheless, regulators need to specify where the realistic risks reside and to quantify them in order to reach a decision over any submission. To achieve this, risk is usually defined by the simple formula: $RISK = HAZARD \times EXPOSURE$. The hazard term relates to the severity of the unwanted change (i.e. how unwanted is the undesirable consequence?) whereas the exposure term is a measure of how likely it is that the unwanted outcome will

occur. Risk can be deemed acceptable even when the consequence (hazard) is deemed high, provided that the exposure is negligible. Equally, a risk can be deemed acceptable when the exposure is high but the hazard (the consequence) is considered of minor importance. Such decisions invariably involve a degree of subjectivity. In the case of gene flow from GM crops (e.g. Anon 2002a; Anon 2003) the risks relate to a variable number of potential ecological and economic hazards (Smyth *et al.* 2002; Strategy Unit 2003) and depend on the features of both the crop and the transgene. The resulting risk assessment procedure is complex. It breaks down into the initial process of identifying and evaluating the hazards, and an evaluation of exposure for hazards deemed to have significance. An extensive volume outlining this risk assessment process has been published recently (Poppy & Wilkinson 2005).

2. European Agriculture and GM Crops

The present review is designed to inform DEFRA about GM crops in a broad European context, and it is therefore necessary to consider the precise definition of 'Europe'. In addition to the 25 states that are now full members of the EU, it is important not to overlook the other overseas territories administered by various EU governments. These regions have a variety of legal relationships with the EU and may only be subject to some areas of EU law (Table 2.1).

Two parts of the treaty of the European Union deal with special relationships: Article 299 sets out the territories to which the treaty applies, supplemented by the accession treaties; and Articles 182-188 and Annex II on association with the non-European countries and territories that have special relations with the member states. For example, according to Article 299 2, Canary Islands (Spain), the Azores and Madeira (Portugal), constitute 'outermost regions' of the EU; provisions of the EC treaty apply there while derogations are allowed. The four French overseas departments (Guadeloupe, Martinique, French Guyana and Réunion) have a similar status in being fully integrated parts of the French state, whereas the other French 'Overseas Territories' and associated dependencies have varying degrees of self government.

The two Spanish enclaves, Ceuta and Melilla are costal towns in Morocco (Figure 2.1). They have a combined area of 32 sq km (12 sq miles). Spain also controls a number of scattered islands along the North African coast, including uninhabited Perejil, which was at the centre of a dispute in 2002 when Moroccan soldiers occupied it before being removed by the Spanish army.

Table 2.1 Territories classified according to level of inclusion in the European Union

This list classifies territories under sovereignty of a EU member state by level of enforceability of EC law in this territory.

EU law fully in force

The part of Cyprus under control of the Republic of Cyprus

Denmark, excluding Faroe Islands and Greenland

Finland, excluding Åland Islands

Metropolitan France

The Netherlands, that is, the European part of the Kingdom of the Netherlands

Portugal, excluding the Azores and Madeira

Spain, excluding Canary Islands, Ceuta and Melilla

The United Kingdom but no other territory under British sovereignty

The totality of the other 17 member states: Austria, Belgium, the Czech Republic, Estonia, Germany, Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Poland, Slovakia, Slovenia, and Sweden

EU law in force, with some exemptions

Åland Islands (Finland)

Guadeloupe, French Guiana, Martinique, Réunion (France)

The Azores, Madeira (Portugal)

Canary Islands, Ceuta, Melilla (Spain)

Gibraltar (UK)

EU law not in force, but some chapters apply

Channel Islands and the Isle of Man (citizens of UK descent are EU citizens; residents do not vote at EU elections)

UK sovereign bases in Cyprus (locals are mainly EU citizens by having Cypriot nationality)

EU law not in force but statute of association with the EU

Greenland (Denmark; locals are EU citizens, but do not vote at EU elections)

French Polynesia, French Southern Territories, Mayotte, New Caledonia, Saint-Pierre and Miquelon, Wallis and Futuna (France; EURATOM Treaty does apply; locals are EU citizens, and do vote at EU elections)

Aruba and Netherlands Antilles (The Netherlands; locals are EU citizens, but do not vote at EU elections)

Anguilla, Bermuda, British Antarctic Territory, British Indian Ocean Territory, British Virgin Islands, Cayman Islands, Falkland Islands, Montserrat, Pitcairn Islands, Saint Helena and Dependencies, South Georgia and the South Sandwich Islands, Turks and Caicos Islands (UK; since 21 May 2002 locals are British citizens and hence EU citizens, however do not vote in EU elections)

EU law not enforceable at all

The part of Cyprus under *de facto* control of the Turkish Republic of Northern Cyprus (locals with Republic of Cyprus nationality are EU citizens, and are entitled to vote at EU elections)

Faroe Islands (Denmark; locals are not EU citizens)

Unclear

Buffer zone in Cyprus

Scattered Islands in the Indian Ocean, Clipperton Island (France; unpopulated)



Figure 2.1 Positions of the two Spanish enclaves on the coast of Morocco
(Source: BBC)

Full EU law applies in Ceuta and Melilla, but some exceptions have been made by a protocol annexed to the treaty of accession of Spain to the Communities (notably agricultural policy and fisheries policy do not apply there).

The relevance of these territories to the present review is that they greatly extend the range of climatic conditions within 'Europe' and therefore significantly increase the diversity of crops to be included in the analysis.

2.1 Review of global GM Field Trials

In order to evaluate the current and future status of GM technology of European crops it is necessary to assess the development and trialling of new GM crop lines. Online databases of field trial applications on a global scale have been examined to determine the current level of commercial interest in a crop and the likelihood of release in the next 5-10 years.

Table 2.2 lists 172 crops for which field trial applications have been made in 31 countries. The data show that most applications (>6000) have been made to trial maize/corn (*Zea mays*). Potato ranks second (1223 applications) with oilseed rape coming a close third (1217), followed by soybean (1022) and cotton (962). Tomatoes, wheat, alfalfa/lucerne, tobacco and rice are also in the top ten, based on applications. US-based biotech industries dominate the majority of applications, with the private sector tending to focus on annual crops rather than perennials. The public sector (Universities and Research Institutes) appears to have an equal if not greater interest in perennials and more specifically tree crops (Table 2.2).

Table 2.2 Global GM field trial applications

Species are listed alphabetically. Information on the source of the application, i.e. the public or the private sector, is made where possible. Applicant details were not always available (No data). Private sector refers to biotech companies, Public Sector to Universities and Research Institutes. Sources of funding for applications were not analysed; thus the division into Public and Private may not be wholly accurate.

	Crop		Field Trial Applications					
			Total	Private Sector	Public Sector	No details	No. of countries	Location
1	Kiwi	<i>Actinidia deliciosa</i>	14	9	5	0	2	Italy, New Zealand
2	Velvet bentgrass	<i>Agrostis canina</i>	1	0	1	0	1	USA
3	Bentgrass	<i>Agrostis</i> spp.	2	2	0	0	1	Japan
4	Creeping bentgrass	<i>Agrostis stolonifera</i>	169	102	67	0	2	Canada, USA
5	Onion	<i>Allium cepa</i>	13	8	5	0	2	New Zealand, USA
6	Leek	<i>Allium porrum</i>	1	0	1	0	1	New Zealand
7	Allegheny service berry	<i>Amelanchier laevis</i>	1	1	0	0	1	USA
8	Pineapple	<i>Ananas comosus</i>	10	0	10	0	3	Australia, Mexico, USA (Hawaii)
9	Lily	<i>Anthurium andraeanum</i>	7	0	7	0	2	New Zealand, USA (Hawaii)
10	Arabidopsis	<i>Arabidopsis</i> spp.	2	0	2	0	2	Mexico, USA
11	Thale Cress	<i>Arabidopsis thaliana</i>	20	0	20	0	2	Sweden, USA
12	Peanut / groundnut	<i>Arachis hypogaea</i>	35	6	29	0	2	China, USA
13	Asparagus	<i>Asparagus officinalis</i>	6	0	6	0	1	New Zealand
14	Belladonna/Deadly nightshade	<i>Atropa belladonna</i>	7	0	7	0	1	USA
15	Oat	<i>Avena sativa</i>	2	0	2	0	1	USA
16	Begonia	<i>Begonia semperflorens</i>	3	0	3	0	1	USA
17	Spinach beet	<i>Beta vulgaris</i> subsp. <i>cicla</i>	1	0	1	0	1	Germany
18	Sea beet	<i>Beta vulgaris</i> subsp. <i>maritima</i>	1	0	1	0	1	Germany
19	Sugar beet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	284	269	15	0	20	Argentina, Belgium, Canada, Chile, Czech Republic, Denmark, Finland, France, Germany, Greece, Hungary, Ireland, Italy, Japan, Netherlands, New Zealand, Spain, Sweden, UK, USA
20	Beet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	177	176	1	0	7	France, Greece, Netherlands, Spain, Sweden, UK, USA
21	Fodder beet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	29	28	1	0	5	Belgium, Denmark, France, Italy, Spain
22	Silver birch	<i>Betula pendula</i>	3	0	3	0	1	Finland
23	Birch	<i>Betula</i> spp.	1	0		1	1	China
24	Ethiopian mustard	<i>Brassica carinata</i>	1	0	1	0	1	Canada
25	Indian mustard	<i>Brassica juncea</i>	6	5	1	0	4	Australia, Belgium, Canada, USA
26	Canola/Oilseed rape	<i>Brassica napus</i>	1217	1202	15	0	18	Australia, Belgium, Canada, Chile, Czech Republic, Denmark, Finland, France, Germany, Italy, Ireland, Mexico, Netherlands, New Zealand, Spain, Sweden, UK, USA
27	Swede	<i>Brassica napus</i>	1	1	0	0	1	Netherlands
28	Canola	<i>Brassica napus/rapa?</i>	9	5	4	0	4	India, Italy, Mexico, South Africa
29	Brown mustard	<i>Brassica nigra</i>	14	14	0	0	2	Canada, India
30	<i>B. oleracea</i>	<i>Brassica oleracea</i>	16	14	2	0	1	USA
31	Cauliflower	<i>Brassica oleracea</i>	11	9	2	0	5	Belgium, Canada, Finland, India, Japan
32	Broccoli	<i>Brassica oleracea</i>	9	3	6	0	4	Canada, Finland, Japan, New Zealand

Table 2.2 *continued*

			Field Trial Applications					Location
			Total	Private Sector	Public Sector	No details	No. of countries	
33	Cabbage	<i>Brassica oleracea</i>	4	3	1	0	3	Finland, India, Netherlands
34	Canola/Turnip rape	<i>Brassica rapa</i>	40	39	1	0	3	Australia, Canada, Sweden
35	<i>B. rapa</i>	<i>Brassica rapa</i>	4	0	4	0	1	USA
36	Chinese cabbage	<i>Brassica rapa</i>	1	0	0	1	1	China
37	Turnip	<i>Brassica rapa</i>	1	1	0	0	1	Hungary
38	Brassica	<i>Brassica</i> spp.	3	0	3	0	2	India, USA
39	Sweet pepper	<i>Capsicum annuum</i>	15	13	0	2	4	China, India, Thailand, USA
40	Chilli	<i>Capsicum annuum</i>	5	2	0	3	4	China, India, Korean Republic, Mexico
41	Papaya	<i>Carica papaya</i>	37	1	33	3	7	Australia, Brazil, China, Cuba, Japan, Mexico, USA
42	Safflower	<i>Carthamus tinctorius</i>	15	13	2	0	2	Mexico, USA
43	American chestnut	<i>Castanea dentata</i>	2	0	2	0	1	USA
44	Sweet chestnut	<i>Castanea sativa</i>	1	1	0	0	1	France
45	Chrysanthemum	<i>Chrysanthemum</i> spp.	6	6	0	0	4	Australia, Japan, Netherlands, USA
46	Chicory	<i>Cichorium intybus</i>	43	42	1	0	6	Belgium, France, Italy, Netherlands, UK, USA
47	Watermelon	<i>Citrullus lanatus</i>	15	15	0	0	2	Italy, USA
48	Lime	<i>Citrus aurantiifolia</i>	1	0	1	0	1	USA (Hawaii)
49	Lemon	<i>Citrus limon</i>	2	0	2	0	2	Italy, Mexico
50	Sweet orange	<i>Citrus sinensis</i>	1	0	1	0	1	Spain
51	Citrange	<i>Citrus sinensis</i> X <i>Poncirus trifoliata</i>	1	1	0	0	1	USA
52	Citrus	<i>Citrus</i> spp.	1	1	0	0	1	Spain
53	Grapefruit	<i>Citrus x paradisi</i>	6	6	0	0	1	USA
54	Coffee	<i>Coffea arabica</i>	3	0	3	0	1	USA (Hawaii)
55	Robusta coffee	<i>Coffea canephora</i>	1	0	1	0	1	France (Guyane)
56	Cucurbit	<i>Cucumis melo</i>	108	98	8	2	1	USA
57	Melon	<i>Cucumis melo</i>	18	15	1	2	6	Egypt, France, Italy, Japan, Mexico, Spain
58	Cantaloupe	<i>Cucumis melo</i>	2	1	0	1	2	Egypt, Spain
59	Cucurbit	<i>Cucumis melo</i> / <i>Cucurbita pepo</i>	17	17	0	0	1	USA
60	Cucurbit	<i>Cucumis melo</i> / <i>Cucurbita pepo</i> / <i>Lycopersicon esculentum</i>	1	0	1	0	1	USA
61	Cucumber	<i>Cucumis sativus</i>	35	29	5	1	3	Egypt, Japan, USA
62	Cucurbit/Squash	<i>Cucurbita pepo</i>	73	58	14	1	5	Egypt, France, Italy, Mexico, Spain
63	Courgette	<i>Cucurbita pepo</i>	16	16	0	0	1	Mexico
64	Squash	<i>Cucurbita texana</i>	3	0	3	0	1	USA
65	Bermudagrass	<i>Cynodon dactylon</i>	14	0	14	0	1	USA
66	Tamarillo/Tree tomato	<i>Cyphomandra betacea</i>	1	1	0	0	1	New Zealand
67	Carrot	<i>Daucus carota</i>	18	3	14	1	3	Brazil, Netherlands, USA
68	Orchid	<i>Dendrobium</i> spp.	3	0	3	0	1	USA (Hawaii)
69	Carnation	<i>Dianthus caryophyllus</i>	41	41	0	0	4	Australia, Japan, Mexico, Netherlands
70	Persimmon	<i>Diospyros virginiana</i>	8	0	8	0	1	USA
71	Murray red gum	<i>Eucalyptus camaldulensis</i>	8	8	0	0	3	Spain, UK, USA

Table 2.2 *continued*

	Crop		Field Trial Applications					
			Total	Private Sector	Public Sector	No details	No. of countries	Location
72	Tasmanian blue gum	<i>Eucalyptus globulus</i>	1	1	0	0	1	Portugal
73	Flooded gum	<i>Eucalyptus grandis</i>	23	23	0	0	3	Spain, UK, USA
74	Eucalyptus	<i>Eucalyptus</i> spp.	3	1	0	3	3	Brazil, South Africa, Japan
75	Lisianthus	<i>Eustoma grandiflorum</i>	1	1	0	0	1	New Zealand
76	Tall fescue	<i>Festuca arundinacea</i>	23	16	7	0	2	France, USA
77	Strawberry	<i>Fragaria ananassa</i>	47	38	7	2	4	Canada, Japan, South Africa, USA
78	Wild strawberry	<i>Fragaria vesca</i>	1	0	1	0	1	Italy
79	Strawberry	<i>Fragaria vesca</i> X <i>Fragaria ananassa</i>	6	0	6	0	2	Italy, Spain
80	Strawberry	<i>Fragaria virginiana</i> X <i>F. chiloensis</i>	1	0	1	0	1	UK
81	Gladiolus	<i>Gladiolus</i> spp.	7	0	7	0	1	USA
82	Soybean	<i>Glycine max</i>	1022	1022	0	0	21	Argentina, Belize, Bolivia, Brazil, Canada, Chile, China, Costa Rica, Dominican Republic, France, Germany, Indonesia, Italy, Japan, Mexico, Puerto Rico, Romania, South Africa, Spain, USA, Uruguay
83	Cotton	<i>Gossypium hirsutum</i>	962	942	1	19	21	Argentina, Australia, Belize, Bolivia, Brazil, Canada, China, Colombia, Costa Rica, France, Ghana, Greece, India, Indonesia, Japan, Mexico, Pakistan, South Africa, Thailand, USA, Zimbabwe
84	Sunflower	<i>Helianthus annuus</i>	52	43	4	5	5	Argentina, France, Netherlands, Spain, USA
85	Barley	<i>Hordeum vulgare</i>	73	38	35	0	8	Australia, Canada, Finland, Hungary, Iceland, New Zealand, UK, USA
86	Sweet potato	<i>Ipomoea batatas</i>	16	11	3	2	4	Cuba, Kenya, USA, Venezuela
87	Walnut	<i>Juglans</i> spp.	15	0	15	0	1	USA
88	Lettuce	<i>Lactuca sativa</i>	94	85	9	0	5	Australia, France, Italy, Japan, USA
89	Lentils	<i>Lens culinaris</i>	1	1	0	0	1	Canada
90	Lily	<i>Lilium longiflorum</i>	1	1	0	0	1	Belgium
91	Statice	<i>Limonium</i> spp.	3	3	0	0	1	Italy
92	Flax	<i>Linum usitatissimum</i>	32	8	24	0	3	Canada, Mexico, Sweden
93	Sweetgum	<i>Liquidambar styraciflua</i>	25	25	0	0	1	USA
94	Italian ryegrass	<i>Lolium multiflorum</i>	2	2	0	0	1	USA
95	Perennial ryegrass	<i>Lolium perenne</i>	8	2	6	0	2	Netherlands, USA
96	Narrow-leaved lupin	<i>Lupinus angustifolius</i>	7	0	7	0	1	Australia
97	Tomato	<i>Lycopersicon esculentum</i>	744	717	19	8	20	Argentina, Australia, Brazil, Canada, Chile, China, Egypt, France, Greece, Guatemala, India, Italy, Japan, Mexico, Netherlands, Portugal, Spain, Thailand, UK, USA
98	Apple	<i>Malus domestica</i>	48	15	32	1	8	Argentina, Belgium, Germany, Netherlands, New Zealand, Sweden, UK, USA
99	Paradise apple	<i>Malus pumila</i>	2	1	1	0	2	Netherlands, UK
100	Cassava	<i>Manihot esculentum</i>	6	2	4	0	1	USA
101	Alfalfa/Lucerne	<i>Medicago sativa</i>	417	392	22	3	7	Argentina, Belgium, Bulgaria, Canada, Mexico, Spain, USA
102	Barrel clover	<i>Medicago truncatula</i>	2	2	0	0	1	USA
103	Peppermint	<i>Mentha x piperita</i>	3	2	1	0	1	USA
104	Banana / plantain	<i>Musa</i> spp.	7	3	3	1	3	Mexico, Philippines, USA
105	Watercress	<i>Nasturtium officinale</i>	1	0	1	0	1	USA

Table 2.2 *continued*

	Crop		Field Trial Applications					
			Total	Private Sector	Public Sector	No details	No. of countries	Location
106	Nicotiana	<i>Nicotiana attenuata</i>	7	0	7	0	1	USA
107	Tobacco	<i>Nicotiana benthamiana</i>	2	2	0	0	1	USA
108	Tobacco	<i>Nicotiana sylvestris</i>	2	0	2	0	1	USA
109	Tobacco	<i>Nicotiana tabacum</i>	374	344	28	2	16	Australia, Brazil, Bulgaria, Canada, Finland, France, Germany, Hungary, India, Italy, Japan, Korean Republic, Mexico, Spain, UK, USA
110	Olive	<i>Olea europea</i>	2	0	2	0	1	Italy
111	Rice	<i>Oryza sativa</i>	323	295	18	10	13	Argentina, Brazil, Canada, China, France, India, Italy, Japan, Mexico, Philippines, Spain, Thailand, USA
112	Marigold	<i>Osteospermum ecklonis</i>	7	2	5	0	2	Italy, USA
113	Poppy - oilseed	<i>Papaver somniferum</i>	2	0	2	0	1	Australia
114	Guayule	<i>Parthenium argentatum</i>	1	0	1	0	1	USA
115	Bahia grass	<i>Paspalum notatum</i>	5	0	5	0	1	USA
116	Scented Pelargonium	<i>Pelargonium odoratissimum</i>	1	0	1	0	1	Italy
117	Pelargonium	<i>Pelargonium</i> spp.	9	8	1	0	2	Italy, USA
118	Avocado	<i>Persea americana</i>	1	0	1	0	1	USA
119	Petunia	<i>Petunia petunia</i> X <i>P. hybrida</i>	29	25	4	0	2	Germany, USA
120	Petunia	<i>Petunia</i> spp.	2	1	1	0	2	Japan, New Zealand
121	Canary grass	<i>Phalaris canariensis</i>	1	0	0	1	1	Canada
122	Norway spruce	<i>Picea abies</i>	3	1	2	0	2	Finland, New Zealand
123	Spruce	<i>Picea</i> spp.	1	0	1	0	1	USA
124	Pine	<i>Pinus radiata</i>	9	9	0	0	1	New Zealand
125	Pine	<i>Pinus</i> spp.	63	62	1	0	2	New Zealand, USA
126	Scots pine	<i>Pinus sylvestris</i>	2	0	2	0	1	Finland
127	Pea	<i>Pisum sativum</i>	56	23	31	2	6	Australia, Canada, Germany, New Zealand, UK, USA
128	Kentucky bluegrass	<i>Poa pratensis</i>	38	28	10	0	1	USA
129	Meadow grass	<i>Poa pratensis</i> X <i>Poa arachnifera</i>	3	3	0	0	1	USA
130	Grey poplar	<i>Populus alba</i> X <i>P. tremula</i>	8	1	7	0	3	France, Spain, UK
131	European aspen	<i>Populus alba</i> X <i>P. tremula</i>	4	0	4	0	3	France, Germany, Norway
132	Poplar	<i>Populus deltoides</i>	143	48	94	1	5	Canada, France, Germany, UK, USA
133	Black poplar	<i>Populus nigra</i>	1	0	0	1	1	China
134	Poplar	<i>Populus</i> spp.	6	4	0	2	2	Belgium, China
135	Poplar/Spruce	<i>Populus</i> spp. / <i>Picea</i> spp.	1	0	1	0	1	USA
136	Hybrid aspen	<i>Populus tremula</i> X <i>P. tremuloides</i>	1	0	1	0	1	Sweden
137	Sweet cherry	<i>Prunus avium</i>	3	0	3	0	1	Italy
138	European plum	<i>Prunus domestica</i>	6	0	6	0	1	USA
139	Cherry / Plum	<i>Prunus domestica</i>	4	0	4	0	2	Italy, Spain
140	Russian wildrye	<i>Psathyrostachys juncea</i> (<i>Elymus junceus</i>)	5	5	0	0	1	USA
141	Pear	<i>Pyrus communis</i>	6	3	3	0	2	Sweden, USA

Table 2.2 *continued*

	Crop		Field Trial Applications					
			Total	Private Sector	Public Sector	No details	No. of countries	Location
142	Wild radish	<i>Raphanus raphanistrum</i>	2	0	2	0	1	France
143	Rhododendron	<i>Rhododendron</i> spp.	8	0	8	0	1	USA
144	Rose	<i>Rosa hybrida</i>	5	5	0	0	2	Australia, USA
145	Raspberry	<i>Rubus idaeus</i>	17	3	14	0	2	Italy, USA
146	Sugarcane	<i>Saccharum officinarum</i>	65	47	11	7	6	Australia, Brazil, Cuba, Egypt, South Africa, USA
147	African violet	<i>Saintpaulia ionantha</i>	1	1	0	0	1	Netherlands
149	White mustard	<i>Sinapis alba</i>	1	0	0	1	1	Canada
150	Eggplant/Brinjal/Aubergine	<i>Solanum melongena</i>	20	4	16	0	3	India, Italy, USA
151	Black nightshade	<i>Solanum nigrum</i>	1	0	1	0	1	Germany
152	Potato	<i>Solanum tuberosum</i>	1223	1083	113	27	31	Argentina, Australia, Austria, Belgium, Bolivia, Brazil, Canada, China, Cuba, Czech Republic, Denmark, Egypt, Finland, France, Germany, Hungary, India, Italy, Japan, Mexico, Netherlands, New Zealand, Peru, Poland, Portugal, South Africa, Spain, Sweden, Sw
153	Sorghum	<i>Sorghum bicolor</i>	1	0	1	0	1	USA
154	St. Augustine grass	<i>Stenotaphrum secundatum</i>	18	18	0	0	1	USA
155	African/Cape marigold	<i>Tagetes erecta</i>	9	0	9	0	1	Italy
156	Torenia	<i>Tourenia fourmieri</i>	2	2	0	0	1	Japan
157	Hare's foot clover	<i>Trifolium arvense</i>	5	4	1	0	1	New Zealand
158	White clover	<i>Trifolium repens</i>	4	1	3	0	1	Australia
159	Subterranean clover	<i>Trifolium subterraneum</i>	9	2	7	0	3	Canada, New Zealand
160	Wheat	<i>Triticum aestivum</i>	451	429	13	9	13	Argentina, Australia, Belgium, Canada, Egypt, Germany, Hungary, Italy, Japan, Mexico, Spain, UK, USA
161	Wheat, durum	<i>Triticum turgidum</i> ssp <i>durum</i>	3	0	3	0	1	Italy
162	American elm	<i>Ulmus americana</i>	3	0	3	0	1	USA
163	Blueberry	<i>Vaccinium</i> spp.	1	0	1	0	1	USA
164	Cranberry	<i>Vaccinium</i> spp.	1	0	1	0	1	USA
165	Adzuki bean	<i>Vigna angularis</i>	1	1	0	0	1	Japan
166	Grape	<i>Vitis berlandieri</i> X <i>V. riparia</i>	1	0	1	0	1	France
167	Grape	<i>Vitis berlandieri</i> X <i>V. rupestris</i>	1	0	1	0	1	France
168	Grape - sand grape	<i>Vitis rupestris</i>	1	0	1	0	1	France
169	Grape	<i>Vitis vinifera</i>	60	32	18	10	6	Australia, Canada, France, Germany, Italy, USA
170	Grape	<i>Vitis vinifera</i> X <i>V. berlandieri</i>	3	0	3	0	1	France
171	Corn/maize	<i>Zea mays</i>	6106	6066	19	21	31	Argentina, Austria, Belgium, Brazil, Canada, Chile, China, Costa Rica, Czech Republic, Denmark, Egypt, France, Germany, Greece, Honduras, Hungary, Indonesia, Italy, Japan, Mexico, Netherlands, New Zealand, Philippines, Portugal, Puerto Rica, Serbia & Mont, UK
172	Zoysiagrass	<i>Zoysia matrella/japonica</i>	2	2	0	0	1	Japan

The Information Systems for Biotechnology (ISB) database of US field trials and commercialised, deregulated crops at Virginia Tech (<http://www.isb.vt.edu>) provides information on the transgene phenotype in each application. Thus it is possible to make some assessment of the more commonly trialled and commercialised transgenic traits in the US.

Table 2.3 Phenotype categories of GM field trial applications in the US

*Other category includes fertility/sterility traits; novel/pharmaceutical protein production; altered gene expression, growth habit or flowering time; tolerance to stress, drought or heavy metals.

Source: Information Systems for Biotechnology Database, Virginia Tech.

Phenotype category	Number of applications
Herbicide tolerance	3820
Insect resistance	3250
Product quality	2497
Agronomic properties	1099
Virus resistance	886
Other*	673
Fungal resistance	661
Marker gene	627
Bacterial resistance	115
Nematode resistance	32

The numbers of applications made in each phenotype category listed in the ISB database clearly demonstrates the prevalence of herbicide tolerance and insect resistance in the development of GM crops (Table 2.3). Agronomic properties and product quality are third and fourth in the ranking.

Table 2.4 Phenotype categories of deregulated, commercial GM crops in the US

Source: Information Systems for Biotechnology Database, Virginia Tech.

Phenotype category	Applications	Approved	Withdrawn	Pending	Void
Herbicide tolerance	45	32	12	1	
Insect resistance	36	23	11	2	
Product quality	20	13	3	2	2
Virus resistance	11	6	3	2	
Agronomic properties	8	6	2		
Other	1	1			
Fungal resistance	0				
Marker gene	0				
Bacterial resistance	0				
Nematode resistance	0				

The small numbers of trials for crops with bacterial and nematode resistance will relate to the specificity of these problems to some crops. For example, applications for trials of material expressing nematode resistance have only been made for soybean, pineapple, carrot, tomato, walnut, grape and tobacco.

Of the commercialised GM crops in the US, i.e. those crops deregulated and no longer under the control of APHIS, the same pattern of transgenic traits is apparent (Table 2.4). Equally, the range of commercialised crops in each phenotype category reflects the focus on herbicide tolerance. This trait has been engineered into seven commercially available lines, insect resistance, product quality, agronomic traits into four lines, and virus resistance into only three.

Table 2.5 Range of US deregulated GM crops according to phenotype category

Source: Information Systems for Biotechnology Database, Virginia Tech.

Phenotype category	Deregulated cultivars
Herbicide tolerance	Maize 11 Oilseed rape 7 Cotton 5 Soybean 4 Beet 3 Rice 1 Alfalfa 1
Insect resistance	Maize 12 Cotton 5 Potato 5 Tomato 1
Product quality	Tomato 10 Tobacco 1 Soybean 1 Oilseed rape 1
Agronomic properties	Maize 3 Flax 1 Oilseed rape 1 Chicory 1
Virus resistance	Potato 3 Squash 2 Papaya 1
Other	Oilseed rape 1

2.1.1 Summary of global GM field trials

Nearly 15,500 field trial applications have been made for 172 crops across 31 countries. The highest-ranking crops, based on applications are:

- 1. Maize/corn (6106)**
- 2. Potato (1223)**
- 3. Oilseed rape (1217)**
- 4. Soybean (1022)**
- 5. Cotton (962)**
- 6. Tomatoes (744)**
- 7. Wheat (451)**
- 8. Alfalfa/Lucerne (417)**
- 9. Tobacco (374)**
- 10. Rice (323)**

2.2 Review of European crop production

Data for the 2004 harvest have been obtained from the EU Statistics database Eurostat (http://epp.eurostat.cec.eu.int/portal/page?_pageid=1090,1&_dad=portal&_schema=PORTAL). Data on individual countries have been consolidated into a list of 133 crops and groups of crops. These have been ranked in order of area under production in both Europe as a geographical and political entity i.e. mainland Europe and the EU25 (Table 2.6).

These data identify common wheat, barley, maize, oilseed rape and durum wheat as the top ranking crops in Europe in terms of acreage. Pasture and meadows also rank very highly in terms of area, indicative of the mixed nature of European agriculture. Of the top twenty crop species, only GM rye have not reached field trials (Table 2.6), although GM rye has been grown in greenhouse tests (Wieser *et al.* 2005).

Specific definitions for the terms used in Eurostat, with regard to pasture and fodder crops, have been sought from Eurostat in London and Luxembourg but without result. Similarly, unsuccessful enquiries were also made with regard to the groupings of minority crops (e.g. Other oilseeds). Such groups occur at an artificially high position in the rank order as individual acreages have been combined. The combination of data in this way therefore does not provide an accurate list of crops under cultivation in individual countries. Without complete knowledge of which crops are being grown in which countries, identifying potential gene escape to the native flora becomes more difficult.

Table 2.6 Crops grown in Europe

Ranking is based on acreage. Definitions: EU25 – Belgium, Czech Republic, Denmark, Germany, Estonia, Greece, Spain, France, Ireland, Italy, Cyprus, Latvia, Lithuania, Luxembourg, Hungary, Malta, Netherlands, Austria, Poland, Portugal, Slovenia, Slovakia, Finland, Sweden and UK. Europe – geographical Europe (EU25 plus data for Bulgaria, Croatia, Romania, Turkey, Iceland, Norway, Albania, Bosnia & Herzegovina and Macedonia where available). * GM field trial application(s) made for this crop; (*) GM trial application(s) made for a closely related species.

GM Field Trials	Crop		Europewide Totals 1000ha	Europe rank	EU25 Totals 1000ha	EU25 rank
*	Wheat	<i>Triticum aestivum</i>	25673.540	1	23276.271	1
	Permanent pasture		23221.136	2	19154.573	2
*	Barley	<i>Hordeum vulgare</i>	13406.994	3	12937.664	3
	Permanent meadows		11038.173	4	9558.198	4
*	Grain maize	<i>Zea mays</i>	9761.463	5	6489.338	6
	Perennial green fodder		9514.773	6	9141.306	5
	Annual green fodder		5090.719	7	4910.824	7
*	Forage maize	<i>Zea mays</i>	4675.841	8	4639.589	8
*	Rape	<i>Brassica napus</i>	4487.458	9	4436.689	9
	Temporary grasses		4214.163	10	4214.163	10
*	Durum wheat	<i>Triticum turgidum</i>	4048.791	11	4044.749	11
*	Sunflower seed	<i>Helianthus annuus</i>	3184.321	12	2201.892	15
*	Oats	<i>Avena sativa</i>	2904.393	13	2673.019	13
	Rye	<i>Secale cereale</i>	2754.255	14	2727.872	12
	Triticale		2481.834	15	2453.905	14
*	Potatoes	<i>Solanum tuberosum</i>	2464.768	16	2174.258	17
*	Sugar beet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	2223.095	17	2200.721	16
*	Wine grapes (juice and/or wine making)	<i>Vitis vinifera</i>	2196.548	18	1962.113	18
*	Lucerne	<i>Medicago sativa</i>	2071.942	19	1836.550	19
	Mixed grain other than maslin		1590.117	20	1590.117	20
*	Total olives (table and oil)	<i>Olea europea</i>	1568.289	21	1568.289	21
	Other annual green fodder		1039.067	22	895.424	22
*	Field peas	<i>Pisum sativum</i>	733.920	23	715.481	23
*	Clover and mixtures	<i>Trifolium</i> spp.	725.360	24	671.797	24
	Other oil seeds (poppy, mustard, safflower, cotton, earth almond, sesame, groundnut)		689.241	25	641.146	25
*	Apples (including cider apples)	<i>Malus domestica</i>	600.090	26	472.505	27
	Other dried pulses		477.115	27	476.730	26
*	Cotton seed	<i>Gossypium hirsutum</i>	463.900	28	463.900	28
*	Other legumes (sainfoin, sweet clover)		431.423	29	367.926	32
*	Rice	<i>Oryza sativa</i>	427.919	30	423.831	29
	Broad and field beans	<i>Vicia faba</i>	406.832	31	391.876	30
*	Soya bean	<i>Glycine max</i>	395.025	32	273.358	33
	Temporary grazings		370.922	33	370.922	31
*	Tomatoes	<i>Lycopersicon esculentum</i>	311.360	34	270.306	34
	Kidney beans	<i>Phaseolus vulgaris</i>	224.978	35	69.808	66
	Buckwheat, millet, canary seed (other cereals)		217.314	36	213.854	35
	Other industrial crops		201.145	37	158.947	37
*	Carrots	<i>Daucus carota</i>	199.404	38	111.469	47
	Other dried pulses (e.g. lathyrus)		198.861	39	198.616	36

Table 2.6 *continued*

GM Field Trials	Crop		Europe-wide Totals 1000ha	Europe rank	EU25 Totals 1000ha	EU25 rank
*	Plums	<i>Prunus domestica</i>	195.076	40	78.182	61
*	Onions	<i>Allium cepa</i>	172.494	41	157.820	38
*	Cherries, including sour cherries	<i>Prunus avium</i>	158.650	42	135.366	43
	Other root crops		157.597	43	157.197	39
	Vetches	<i>Vicia</i> spp.	153.241	44	153.241	40
*	Fodder beet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	144.622	45	104.283	50
*	Cauliflower and broccoli	<i>Brassica oleracea</i>	143.956	46	142.146	41
*	Flax (straw)	<i>Linum usitatissimum</i>	135.705	47	135.410	42
*	Peas other than field peas (including chick peas)		133.267	48	126.087	46
	Almonds	<i>Prunus dulcis</i>	130.407	49	128.495	44
*	Tobacco raw (including seedlings enclosures)	<i>Nicotiana tabacum</i>	128.819	50	103.692	51
*	Oranges	<i>Citrus sinensis</i>	127.059	51	127.059	45
	Official herbs, aromatic plants, plants for seasoning (e.g. thyme)		118.945	52	109.848	48
*	Pears (including perry pears)	<i>Pyrus communis</i>	110.351	53	104.608	49
*	Table grapes	<i>Vitis vinifera</i>	107.406	54	89.650	59
	Peaches	<i>Prunus persica</i>	105.511	55	96.621	54
*	Sorghum	<i>Sorghum bicolor</i>	103.384	56	94.708	56
*	Cabbage (white)	<i>Brassica oleracea</i>	103.131	57	74.065	64
*	Water melons	<i>Citrullus lanatus</i>	101.493	58	60.842	68
*	Lettuce	<i>Lactuca sativa</i>	100.647	59	99.078	52
*	Oil flax	<i>Linum usitatissimum</i>	99.834	60	98.427	53
(*)	Maslin	<i>Triticum aestivum</i> and <i>Secale cereale</i>	96.131	61	96.131	55
*	Strawberries	<i>Fragaria ananassa</i>	95.428	62	91.794	57
*	Melons	<i>Cucumis melo</i>	95.288	63	89.940	58
	Globe artichokes	<i>Cynara scolymus</i>	82.037	64	82.037	60
*	Lupins	<i>Lupinus angustifolius</i>	76.064	65	76.064	62
*	Turnip rape	<i>Brassica rapa</i>	75.984	66	75.984	63
	Hazelnuts	<i>Corylus avellana</i>	73.359	67	73.253	65
*	Red pepper, capsicum	<i>Capsicum annuum</i>	68.241	68	48.483	73
	Other permanent crops (carob-tree, mulberry-tree, tea, coffee)		64.267	69	63.780	67
	Carobs	<i>Ceratonia siliqua</i>	60.124	70	60.124	69
	Industrial crops (rye-straw, fullers teasel, lavender, hybrid lavender)		57.536	71	24.435	94
	Other root crops (Jerusalem artichoke, sweet potatoes, fodder parsnips, yams, cassava)		54.800	72	54.400	70
	Apricots	<i>Prunus armeniaca</i>	54.128	73	42.509	75
	Asparagus	<i>Asparagus officinalis</i>	53.279	74	53.279	71
	Black currants	<i>Ribes nigrum</i>	48.721	75	48.657	72
*	Lentils	<i>Lens culinaris</i>	45.709	76	45.569	74
	Nectarines	<i>Prunus persica</i> var. <i>nectarina</i>	41.545	77	41.545	76
	Other leafy or stalked vegetables		41.221	78	39.152	78
	Garlic	<i>Allium sativum</i>	40.311	79	33.691	82
*	Chicory	<i>Cichorium intybus</i>	39.802	80	39.802	77
*	Cucumbers	<i>Cucumis sativus</i>	38.339	81	30.912	84
	Chestnuts	<i>Castanea sativa</i>	37.875	82	37.850	79

Table 2.6 *continued*

GM Field Trials	Crop		Europewide Totals 1000ha	Europe rank	EU25 Totals 1000ha	EU25 rank
*	Walnuts	<i>Juglans regia</i>	37.732	83	27.223	89
	Hops	<i>Humulus lupulus</i>	36.543	84	36.493	80
*	Other brassicas	<i>Brassica spp</i>	34.098	85	33.795	81
*	Lemons	<i>Citrus limon</i>	31.676	86	31.676	83
(*)	Gourds	Cucurbitaceae	30.856	87	28.758	87
	Other pulses		30.848	88	30.766	85
*	Kiwi	<i>Actinidia deliciosa</i>	28.944	89	28.920	86
*	Fodder kale	<i>Brassica oleracea</i>	28.004	90	28.004	88
	Spinach	<i>Spinacia oleracea</i>	27.671	91	27.216	90
*	Beetroot	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>	27.241	92	27.101	91
*	Marrows, courgettes	<i>Cucurbita pepo</i>	25.961	93	25.027	92
	Clementines	<i>Citrus reticulata</i> cv. <i>Clementine</i>	24.985	94	24.985	93
*	Leeks	<i>Allium ampeloprasum</i>	23.536	95	22.267	95
*	Egg-plant	<i>Solanum melongena</i>	23.363	96	16.012	100
*	Raspberries	<i>Rubus idaeus</i>	22.007	97	20.328	97
*	Chicory for inulin	<i>Cichorium intybus</i>	20.861	98	20.861	96
	Endive	<i>Cichorium endivia</i>	19.790	99	19.790	98
	Caraway	<i>Carum carvi</i>	17.199	100	17.199	99
*	Brussels sprouts	<i>Brassica oleracea</i>	13.878	101	13.867	101
	Hemp (straw)	<i>Cannabis sativa</i>	13.877	102	12.682	103
	Red currants	<i>Ribes rubrum</i>	13.247	103	13.246	102
	Other soft fruit		11.736	104	11.422	104
*	Turnips	<i>Brassica rapa</i>	10.962	105	10.962	105
	Mandarins	<i>Citrus reticulata</i>	10.325	106	10.325	106
	Other root and tuber vegetables		10.112	107	8.611	108
*	Chicory	<i>Cichorium intybus</i>	8.803	108	8.803	107
(*)	Radishes	<i>Raphanus sativus</i>	8.770	109	7.929	109
*	Turnips for stockfeeding	<i>Brassica rapa</i>	7.691	110	7.691	110
	Celeriac	<i>Apium graveolens</i> var. <i>rapaceum</i>	6.712	111	6.712	111
	Celery	<i>Apium graveolens</i> var. <i>dulce</i>	6.507	112	6.161	112
*	Gherkins	<i>Cucumis sativus</i>	5.351	113	4.394	114
	Figs	<i>Ficus carica</i>	5.219	114	5.218	113
	Other fruit		4.422	115	3.816	116
	Gooseberries	<i>Ribes uva-crispa</i>	4.340	116	4.340	115
*	Kohl-Rabi	<i>Brassica oleracea</i>	3.196	117	3.136	118
	Salsify and scorzonera	<i>Tragopogon porrifolius</i> and <i>Scorzonera hispanica</i>	3.145	118	3.145	117
*	Shallots	<i>Allium cepa</i>	2.487	119	2.487	119
	Other vegetables cultivated for fruit		2.267	120	1.853	120
*	Grapefruit	<i>Citrus x paradisi</i>	1.075	121	1.075	121
	Quinces	<i>Cydonia oblonga</i>	0.696	122	0.571	122
	Other stone fruit		0.292	123	0.292	123
*	Carrots for stockfeeding	<i>Daucus carota</i>	0.149	124	0.149	124
*	Avocados	<i>Persea americana</i>	0.139	125	0.139	125
	Other nuts		0.120	126	0.120	126

Table 2.6 *continued*

GM Field Trials	Crop		Europewide Totals 1000ha	Europe rank	EU25 Totals 1000ha	EU25 rank
	Cultivated mushrooms	<i>Agaricus bisporus</i>	0.088	127	0.088	127
*	Swedes	<i>Brassica napus</i>	0.023	128	0.023	128
	Wild products (truffles, other mushrooms, grape hyacinth-bulbs)		0.000	129	0.000	129
	Satsumas	<i>Citrus reticulata</i>	0.000	130	0.000	130
*	Raisins (in fresh weight)	<i>Vitis vinifera</i>	0.000	131	0.000	131
	Other citrus fruit		0.000	132	0.000	132
*	Limes	<i>Citrus aurantiifolia</i>	0.000	133	0.000	133

2.2.1 Summary of European field crops

The highest-ranking crops based on area under cultivation (excluding aggregates of species) are listed in Table 2.7.

Table 2.7 Summary of European field crops and the development of GM varieties

Rank		GM varieties in field trials	GM varieties commercially available	No GM varieties in field trials
1	wheat	+		
2	barley	+		
3	grain maize		+	
4	forage maize		+	
5	oilseed rape		+	
6	durum wheat	+		
7	sunflowers	+		
8	oats	+		
9	rye			+
10	triticale			+
11	potatoes	+		
12	sugar beet	+		
13	grapes	+		
14	alfalfa/lucerne	+		
15	olives	+		
16	field peas	+		
17	clover	+		
18	apples	+		
19	cotton		+	
20	rice	+		

2.2.2 Overseas departments

Crop production data for the overseas departments of the EU have been more difficult to source, as separate data are not available through Eurostat. Detailed production data have therefore not been obtained, although a list of crop species has been compiled from a variety of web sources (Appendix II) for the following territories: Azores-PT, Madeira-PT, Canary Islands-ES, Guadeloupe-FR, Guyane-FR, Martinique-FR and Réunion-FR. This list contains 55 different crops of which 12 do not occur on the mainland list of crops and thus appear to be unique to the overseas territories (Table 2.8). These include cocoa, coconuts, dates, custard apples, inhame, lychees, mangoes, papaya, passion fruit, pineapples, tea and vanilla. These crops may not be entirely absent from mainland Europe as exhaustive crop data for the mainland have not yet been obtained. Indeed, a small tea crop is now being produced in the UK. Crops such as cocoa in Guyane however, are restricted to the tropics and are unlikely to be cultivated in mainland Europe. Of these 12 additional crops, only two have been through GM field trials: papaya and pineapple. The remaining ten crops, with the addition of figs, rye, tangerines, tea and vanilla do not have transgenic varieties that have reached field trials (Table 2.8).

Trialling of GM crops has not taken place on a large scale in the overseas territories, although a recent report describes the first long-term field trial of GM coffee in Guyane (Perthuis *et al.* 2005). Clones of Robusta coffee (*Coffea canephora*) were transformed for resistance to the lepidopteran coffee leaf miner *Leucoptera coffeella* by the insertion of a synthetic *cry1ac* Bt gene using *A. tumefaciens*. Over a 4-year period six releases of *L. coffeella* were performed and the results showed that the majority of the independent transformed clones displayed much less insect damage than the control.

C. canephora is an insect pollinated, out-crossing crop that will hybridise with related wild species. It is, however, native to central Africa (Zaire, Sudan, Uganda, Tanzania) and all its wild relatives are indigenous to Africa, Madagascar and the

Table 2.8 Crops of Overseas Departments of Europe

Figures where present represent area under cultivation in ha; + indicates that cultivation of a crop occurs on some scale; * indicates that GM field trial applications(s) have been made for this crop.

GM Field Trials	Crop		Guadeloupe	Martinique	Reunion	Guyane	Azores	Madeira	Canary Islands
*	kiwi	<i>Actinidia deliciosa</i>						11	
*	onions	<i>Allium cepa</i>						52	+
*	pineapples	<i>Ananas comosus</i>		+			+		
	custard apple	<i>Anona spp</i>						103	
*	oats	<i>Avena sativa</i>					+		
*	sugarbeet	<i>Beta vulgaris</i> subsp. <i>vulgaris</i>					+		
*	cabbage	<i>Brassica oleracea</i>						60	
*	cauliflower	<i>Brassica oleracea</i>						25	
*	turnip	<i>Brassica rapa</i>						10	
	tea	<i>Camelia sinensis</i>					+		
*	papaya	<i>Carica papaya</i>						2.5	
*	chestnut	<i>Castanea sativa</i>						64	
*	chicory	<i>Cichorium intybus</i>					+		
*	lemons	<i>Citrus limon</i>						87	+
	tangerine	<i>Citrus reticulata</i>						13.5	
*	oranges	<i>Citrus sinensis</i>						117	+
	coconuts	<i>Cocos nucifera</i>							+
	inhame	<i>Colocasia antiquorum</i>						32	
*	melon	<i>Cucumis melo</i>	410						
*	courgettes	<i>Cucurbita pepo</i>							+
*	pumpkin	<i>Cucurbita pepo</i>						20	
*	carrots	<i>Daucus carota</i>						50	+
	figs	<i>Ficus carica</i>							
*	strawberry	<i>Fragaria ananassa</i>						12	
*	cotton	<i>Gossypium hirsutum</i>							+
*	sweet potato	<i>Ipomoea batatas</i>						450	
*	lettuce	<i>Lactuca sativa</i>		+				50	+
	lychees	<i>Litchi chinensis</i>			+				
*	tomatoes	<i>Lycopersicon esculentum</i>						100	+
*	apples	<i>Malus x domestica</i>						195	
	mangoes	<i>Mangifera indica</i>		+					
(*)	manioc (tapioca)	<i>Manihot ultissima</i>				+			
*	bananas	<i>Musa spp.</i>	6000	+		+			+
*	tobacco	<i>Nicotiana tabacum</i>			+				+
*	rice	<i>Oryza sativa</i>				8295			
	passion fruit	<i>Passiflora edulis</i>					+	11.8	
*	avocados	<i>Persea americana</i>		+				85	
	dates	<i>Phoenix dactylifera</i>							+
*	cherry	<i>Prunus avium</i>						22	
*	plum	<i>Prunus domestica</i>						10.5	
	peach	<i>Prunus persica</i>						9	
*	pears	<i>Pyrus communis</i>						89	
*	sugarcane	<i>Saccharum officinarum</i>	14300	+	+	240		125	+
	rye	<i>Secale cereale</i>					+		
*	eggplant	<i>Solanum melongena</i>	+						
*	potatoes	<i>Solanum tuberosum</i>					+	1600	79
	cocoa	<i>Theobroma cacao</i>				+			
*	wheat	<i>Triticum aestivum</i>					+		
	vanilla	<i>Vanilla planifolia</i> and other spp.			+				
*	wine grapes	<i>Vitis vinifera</i>					+		+
*	maize	<i>Zea mays</i>			+	+	+	80	+
	beans						+		+
	beans, French							90	
	beans, green							9	
	flowers		+	+					

Mascarenes (Ellstrand 2003c). In addition, coffee is not listed as a major crop in Guyane (Table 2.8) thus the risks of transgene escape via hybridisation during this trial would be minimal.

2.2.3 Forestry

Forestry production is difficult to quantify, as survey data of forest resources are not collected according to species. This is also true for forest plantations. However, recent global forest surveys show that in most regions of the world, relatively few tree species make up the majority of standing wood volume (FAO 2005). A list of dominant forest species has been compiled for all the countries of Europe from the FAO Forest Resources Assessment, 2000 (FAO 2000). These data are consolidated in Table 2.9 to give a list of species for Europe as a whole. There are 20 species listed of which the majority are native to Europe. Only six species are introduced to Europe, namely *Eucalyptus globulus*, *Larix kaempferi*, *Picea sitchensis*, *Pinus contorta* var. *latifolia*, *Pinus radiata* and *Pseudotsuga menziesii*, the majority of which will account for the planted forest area. The largest areas of natural forest occur in Sweden (26,565 thousand ha), Finland (21,935 thousand ha), France (14,380 thousand ha), Spain (12,466 thousand ha) and Germany (10,740 thousand ha) (FAO 2000). Areas of planted forest are much less in comparison. The largest planted areas occur in the UK (1928 thousand ha), Spain (1904 thousand ha), Bulgaria (969 thousand ha), France (961 thousand ha) and Portugal (834 thousand ha) (Stace 1975; Simmonds 1976; Fehr and Hadley 1980; Smart and Simmonds 1995; Ellstrand 2003b).

Table 2.9 Dominant forest species of Europe

Species list includes tree species of both natural woodland and forest plantations.

	Europewide Totals 1000ha	EU25 Totals 1000ha	
Planted forest	10011	8403	
Natural forest	154445	131062	
Natural woodland and planted species			GM Field Trials
Silver fir	<i>Abies alba</i>		
Sycamore	<i>Acer pseudoplatanus</i>		
Alder	<i>Alnus</i> spp.		
White / Silver birch	<i>Betula pendula</i>		✓
Downy birch	<i>Betula pubescens</i>		
Birch	<i>Betula</i> spp.		✓
Sweet chestnut	<i>Castanea sativa</i>		✓
Tasmanian blue gum	<i>Eucalyptus globulus</i>		✓
Beech	<i>Fagus sylvatica</i>		
Ash	<i>Fraxinus excelsior</i>		
European larch	<i>Larix decidua</i>		
Japanese/hybrid larch	<i>Larix kaempferi</i>		
Norway spruce	<i>Picea abies</i>		✓
Sitka spruce	<i>Picea sitchensis</i>		✓
Lodgepole pine	<i>Pinus contorta</i> var. <i>latifolia</i>		
Corsican pine	<i>Pinus nigra</i> subsp. <i>laricio</i>		
Maritime pine	<i>Pinus pinaster</i>		
Monterey pine	<i>Pinus radiata</i>		✓
Pines	<i>Pinus</i> spp.		✓
Scots pine	<i>Pinus sylvestris</i>		✓
Poplar	<i>Populus</i> spp.		✓
Quaking aspen	<i>Populus tremula</i>		✓
Douglas fir	<i>Pseudotsuga menziesii</i>		
Oak	<i>Quercus</i> spp.		
Cork oak	<i>Quercus suber</i>		
Elm	<i>Ulmus</i> spp.		✓

2.3 Potential gene flow: recipient species and wild relatives of European crops

All crop species on the GM field trials list (Table 2.2) have been assessed with regard to the potential for transgene escape to wild relatives by investigating known hybridisation events with related species (Tutin *et al.* 1964-1980; Stace 2001; Ellstrand 2003b; Jørgensen & Wilkinson 2005). In addition, crop species that have wild relatives native to Europe have been screened to evaluate the potential for further hybridisation events and the subsequent ecological impact with respect to endemism and rarity of related taxa. The extent of known escape and naturalisation of crop species has also been recorded where data are available.

The results identify those crop species with known ability to form spontaneous hybrids with wild species, those crops that may have the potential to hybridise under certain conditions, and those which pose no risk of transgene spread to wild relatives in Europe (Table 2.10).

2.3.1 Crops posing less risk of transgene spread

Of the 172 crops listed in Table 2.10, only 17 appear to pose a low risk of transgene escape via hybridisation with wild species in Europe. These are kiwi, pineapple, papaya, watermelon, Tasmanian blue gum, soybean, sweet potato, lily, tomato, cassava, banana, rice, aubergine, potato, sorghum and maize. Of these 17, only two are in the top ten crop species of Europe, namely maize and potatoes, although rice, soybean and tomato are relatively high-ranking crops (30, 32 and 34 respectively). The remainder of crops posing limited risk are relatively minor, with four being cultivated only in the Overseas Departments (pineapple, papaya, sweet potato and banana) (Table 2.10).

Table 2.10 Crops from GM Field Trials with wild relatives and hybridisation potential in Europe. Rank order of crop species is based on the area under production in 2004 (Table 2.6). Plant family and the geographical origin of crops are shown.

Rank	Risk	Crop		Family	Native to
		Kiwi	<i>Actinidia deliciosa</i>	Actinidaceae	China
	**	Velvet bentgrass	<i>Agrostis canina</i>	Gramineae	W Asia, Europe
	**	Bentgrass	<i>Agrostis</i> spp.	Gramineae	N temperate
	**	Creeping bentgrass	<i>Agrostis stolonifera</i>	Gramineae	Macaronesia, N Africa, Temperate Asia, Europe
41, osd		Onion	<i>Allium cepa</i>	Liliaceae	only cultivated
95	**	Leek	<i>Allium porrum</i>	Liliaceae	only cultivated
		Allegheny service berry	<i>Amelanchier laevis</i>	Rosaceae	E N America
osd		Pineapple	<i>Ananas comosus</i>	Bromeliaceae	Brazil
		Lily	<i>Anthurium andraeanum</i>	Araceae	Tropical America
		Arabidopsis	<i>Arabidopsis</i> spp	Brassicaceae	N temperate
		Thale Cress	<i>Arabidopsis thaliana</i>	Brassicaceae	N temperate
		Peanut	<i>Arachis hypogaea</i>	Leguminosae	South America
	*	Asparagus	<i>Asparagus officinalis</i>	Liliaceae	Europe - N Africa
		Belladonna / Deadly nightshade	<i>Atropa belladonna</i>	Solanaceae	Mediterranean-Eurasia
13,osd	**	Oat	<i>Avena sativa</i>	Gramineae	Mediterranean basin
		Begonia	<i>Begonia semperflorans</i>	Begoniaceae	SE Brazil, NE Argentina
		Spinach beet	<i>Beta vulgaris subsp cicla</i>	Chenopodiaceae	Europe, near East, Asia to China
		Sea beet	<i>Beta vulgaris subsp maritima</i>	Chenopodiaceae	Europe, near East, Asia to China
92	**	Beet	<i>Beta vulgaris subsp vulgaris</i>	Chenopodiaceae	Europe, near East, Asia to China
45	**	Fodder beet	<i>Beta vulgaris subsp vulgaris</i>	Chenopodiaceae	Europe, near East, Asia to China
17,osd	**	Sugar beet	<i>Beta vulgaris subsp vulgaris</i>	Chenopodiaceae	Europe, near East, Asia to China
fs	**	Silver birch	<i>Betula pendula</i>	Betulaceae	Europe
fs	**	Birch	<i>Betula</i> spp.	Betulaceae	N. hemisphere
		Ethiopian mustard	<i>Brassica carinata</i>	Brassicaceae	Europe
		Indian mustard	<i>Brassica juncea</i>	Brassicaceae	Europe, Asia
9	**	Canola/Oilseed rape	<i>Brassica napus</i>	Brassicaceae	Europe
128	**	Swede	<i>Brassica napus</i>	Brassicaceae	Europe
		Canola	<i>Brassica napus/rapa?</i>	Brassicaceae	Europe
		Brown mustard	<i>Brassica nigra</i>	Brassicaceae	Europe, Asia Minor, Iran, Asia and Far East
		B. oleracea	<i>Brassica oleracea</i>	Brassicaceae	Europe
46	**	Broccoli	<i>Brassica oleracea</i>	Brassicaceae	Europe
57,osd	**	Cabbage	<i>Brassica oleracea</i>	Brassicaceae	Europe
46,osd	**	Cauliflower	<i>Brassica oleracea</i>	Brassicaceae	Europe
		<i>B. rapa</i>	<i>Brassica rapa</i>	Brassicaceae	Europe
66	**	Canola/Turnip rape	<i>Brassica rapa</i>	Brassicaceae	Europe
		Chinese cabbage	<i>Brassica rapa</i>	Brassicaceae	Europe
105, osd	**	Turnip	<i>Brassica rapa</i>	Brassicaceae	Europe
		Brassica	<i>Brassica</i> spp	Brassicaceae	Europe
		Chilli	<i>Capsicum annuum</i>	Solanaceae	New World tropics
68	*	Sweet pepper	<i>Capsicum annuum</i>	Solanaceae	New World tropics
osd		Papaya	<i>Carica papaya</i>	Caricaceae	Tropical Americas
	*	Safflower	<i>Carthamus tinctorius</i>	Asteraceae	Turkey, Lebanon, Israel to NW India

Table 2.10 *continued*

Rank	Risk	Crop		Family	Native to
		American chestnut	<i>Castanea dentata</i>	Fagaceae	Asia, introduced to N America
osd,fs	**	Sweet chestnut	<i>Castanea sativa</i>	Fagaceae	Mediterranean-Caucasus
	⊕	Chrysanthemum	<i>Chrysanthemum</i> spp.	Asteraceae	N Africa, Europe-Asia
80,osd	*	Chicory	<i>Cichorium intybus</i>	Asteraceae	Mediterranean
58		Watermelon	<i>Citrullus lanatus</i>	Cucurbitaceae	Tropical and Southern Africa
	*	Lime	<i>Citrus aurantiifolia</i>	Rutaceae	Southeast Asia
86,osd	*	Lemon	<i>Citrus limon</i>	Rutaceae	Southeast Asia
51,osd	*	Sweet orange	<i>Citrus sinensis</i>	Rutaceae	Southeast Asia
		Citrange	<i>Citrus sinensis</i> X <i>Poncirus trifoliata</i>	Rutaceae	Southeast Asia
		Citrus	<i>Citrus</i> spp.	Rutaceae	Southeast Asia
121	**	Grapefruit	<i>Citrus x paradisi</i>	Rutaceae	Southeast Asia
		Coffee	<i>Coffea arabica</i>	Rubiaceae	SW Ethiopia-Sudan
		Robusta coffee	<i>Coffea canephora</i>	Rubiaceae	Zaire, Sudan, Uganda, NW Tanzania
63	*	Cantaloupe	<i>Cucumis melo</i>	Cucurbitaceae	Subgen Melo Africa-India
		Cucurbit	<i>Cucumis melo</i>	Cucurbitaceae	Subgen Melo Africa-India
63,osd	*	Melon	<i>Cucumis melo</i>	Cucurbitaceae	Subgen Melo Africa-India
		Cucurbit	<i>Cucumis melo</i> / <i>Cucurbita pepo</i>	Cucurbitaceae	
		Cucurbit	<i>Cucumis melo</i> / <i>Cucurbita pepo</i> / <i>Lycopersicon esculentum</i>		
81,113	*	Cucumber	<i>Cucumis sativus</i>	Cucurbitaceae	Subgen Cucumis, Asia
93,osd	*	Courgette	<i>Cucurbita pepo</i>	Cucurbitaceae	Tropical America
87,osd	*	Cucurbit/Squash	<i>Cucurbita pepo</i>	Cucurbitaceae	Tropical America
		Squash	<i>Cucurbita texana</i>	Cucurbitaceae	Tropical America
		Bermudagrass	<i>Cynodon dactylon</i>	Chloridoideae	Pan-tropical, extending into warm temperate
		Tamarillo/tree tomato	<i>Cyphomandra betacea</i>	Solanaceae	Tropical America
38,osd	**	Carrot	<i>Daucus carota</i>	Umbelliferae	Mediterranean region
		Orchid	<i>Dendrobium</i> spp	Orchidaceae	Tropical Asia-Australia-Pacific
		Carnation	<i>Dianthus caryophyllus</i>	Caryophyllaceae	Mediterranean
		Persimmon	<i>Diospyros virginiana</i>	Ebenaceae	SE USA
		Murray red gum	<i>Eucalyptus camaldulensis</i>	Myrtaceae	Australia
fs		Tasmanian blue gum	<i>Eucalyptus globulus</i>	Myrtaceae	Australia
		Flooded gum	<i>Eucalyptus grandis</i>	Myrtaceae	Australia
		Lisianthus	<i>Eustoma grandiflorum</i>	Gentianaceae	N America
	**	Tall fescue	<i>Festuca arundinacea</i>	Gramineae	Temperate Asia-Europe, naturalised elsewhere
		Wild strawberry	<i>Fragaria vesca</i>	Rosaceae	N temperate Europe, Asia, Americas
		Strawberry	<i>Fragaria vesca</i> X <i>Fragaria ananassa</i>	Rosaceae	
		Strawberry	<i>Fragaria virginiana</i> X <i>F. chiloensis</i>	Rosaceae	<i>F. chiloensis</i> - Pacific coast of N America, Chile inland at altitudes of up to 1600m. <i>F. virginiana</i> - Rocky Mts, from New Mexico to Alaska.
62,osd	*	Strawberry	<i>Fragaria x ananassa</i>	Rosaceae	N temperate Europe, Asia, Americas
	⊕	Gladiolus	<i>Gladiolus</i> spp	Iridaceae	Africa
32		Soybean	<i>Glycine max</i>	Leguminosae	C & N Asia
28,osd	*	Cotton	<i>Gossypium hirsutum</i>	Malvaceae	Seasonally arid tropics and sub-tropics

Table 2.10 *continued*

Rank	Risk	Crop		Family	Native to
12	*	Sunflower	<i>Helianthus annuus</i>	Asteraceae	Temperate N America
3	**	Barley	<i>Hordeum vulgare</i>	Gramineae	Domesticated from wild races found in SW Asia/near East
osd		Sweet potato	<i>Ipomoea batatas</i>	Convolvulaceae	South America
83	**	Walnut	<i>Juglans</i> ssp.	Juglandaceae	SE Europe - China
59,osd	**	Lettuce	<i>Lactuca sativa</i>	Asteraceae	Mediterranean basin
76		Lentils	<i>Lens culinaris</i>	Leguminosae	Mediterranean basin & SW Asia
		Lily	<i>Lilium longiflorum</i>	Liliaceae	Easter I, Bermuda, Japan
	⊕	Statice	<i>Limonium</i> spp.	Plumbaginaceae	Maritime & arid N hemisphere
47,60	**	Flax	<i>Linum usitatissimum</i>	Linaceae	Eurasia
		Sweetgum	<i>Liquidambar styraciflua</i>	Hamamelidaceae	N-C America
	**	Italian ryegrass	<i>Lolium multiflorum</i>	Gramineae	C&S Europe
	**	Perennial ryegrass	<i>Lolium perenne</i>	Gramineae	Europe
65	**	Narrow-leaved lupin	<i>Lupinus angustifolius</i>	Leguminosae	Mediterranean basin
34,osd		Tomato	<i>Lycopersicon esculentum</i>	Solanaceae	W S America, C America, subtropics of Old World
		Paradise apple	<i>Malus pumila</i>	Rosaceae	Asia Minor, Caucasus, Soviet Central Asia
26,osd	**	Apple	<i>Malus x domestica</i>	Rosaceae	Asia Minor, Caucasus, Soviet Central Asia
osd		Cassava	<i>Manihot esculentum</i>	Euphorbiaceae	S & C America
19	**	Alfalfa/Lucerne	<i>Medicago sativa</i>	Leguminosae	SW Asia
	**	Barrel clover	<i>Medicago truncatula</i>	Leguminosae	Europe-Mediterranean-S Africa
		Peppermint	<i>Mentha x piperita</i>	Labiatae	Europe
osd		Banana / plantain	<i>Musa</i> spp.	Musaceae	SE Asia and Pacific
		Watercress	<i>Nasturtium officinale</i>	Brassicaceae	Europe
		Nicotiana	<i>Nicotiana attenuata</i>	Solanaceae	W N America
		Tobacco	<i>Nicotiana benthamiana</i>	Solanaceae	Australia
		Tobacco	<i>Nicotiana sylvestris</i>	Solanaceae	S America
50,osd	*	Tobacco	<i>Nicotiana tabacum</i>	Solanaceae	S & C America
21	**	Olive	<i>Olea europea</i>	Oleaceae	Mediterranean basin
30,osd		Rice	<i>Oryza sativa</i>	Gramineae	Asia
		Marigold	<i>Osteospermum ecklonis</i>	Asteraceae	Southern Africa
		Poppy - oilseed	<i>Papaver somniferum</i>	Papaveraceae	W Mediterranean - Asia
		Guayule	<i>Parthenium argentatum</i>	Asteraceae	Texas-N Mexico
		Bahia grass	<i>Paspalum notatum</i>	Panicaceae	New World
		Scented Pelargonium	<i>Pelargonium odoratissimum</i>	Geraniaceae	Southern Africa
	⊕	Pelargonium	<i>Pelargonium</i> spp.	Geraniaceae	Southern Africa
125, osd	*	Avocado	<i>Persea americana</i>	Lauraceae	Americas
		Petunia	<i>Petunia petunia</i> X <i>P. hybrida</i>	Solanaceae	(Sub)tropical S America
	⊕	Petunia	<i>Petunia</i> ssp.	Solanaceae	(Sub)tropical S America
	*	Canary grass	<i>Phalaris canariensis</i>	Gramineae	W Mediterranean
fs	**	Norway spruce	<i>Picea abies</i>	Pinaceae	N & C Europe
fs	**	Spruce	<i>Picea</i> ssp.	Pinaceae	Cool N hemisphere
fs	*	Monterey pine	<i>Pinus radiata</i>	Pinaceae	California
fs	**	Pine	<i>Pinus</i> spp.	Pinaceae	N temperate
fs	**	Scots pine	<i>Pinus sylvestris</i>	Pinaceae	Eurasia
23	*	Pea	<i>Pisum sativum</i>	Leguminosae	Mediterranean, near East

Table 2.10 *continued*

Rank	Risk	Crop		Family	Native to
	**	Common meadow grass / Kentucky bluegrass	<i>Poa pratensis</i>	Gramineae	Mediterranean-Eurasia
	**	Meadow grass	<i>Poa pratensis</i> X <i>Poa arachnifera</i>	Gramineae	
		European aspen	<i>Populus alba</i> X <i>P. tremula</i>	Salicaceae	
		Grey poplar	<i>Populus alba</i> X <i>P. tremula</i>	Salicaceae	
		Cottonwood	<i>Populus deltoides</i>	Salicaceae	E N America
		Black poplar	<i>Populus nigra</i>	Salicaceae	Europe, W Asia
fs		Poplar	<i>Populus</i> spp.	Salicaceae	N temperate
		Poplar/Spruce	<i>Populus</i> spp. / <i>Picea</i> spp.	Salicaceae	
		Hybrid aspen	<i>Populus tremula</i> X <i>P. tremuloides</i>	Salicaceae	
42,osd	*	Sweet cherry	<i>Prunus avium</i>	Rosaceae	Western Asia
		Cherry / Plum	<i>Prunus domestica</i>	Rosaceae	Europe
40,osd	**	European plum	<i>Prunus domestica</i>	Rosaceae	Europe
	*	Russian wildrye	<i>Psathyrostachys juncea</i>	Gramineae	Eurasia
53,osd	**	Pear	<i>Pyrus communis</i>	Rosaceae	Asia Minor, Caucasus, Soviet Central Asia
		Wild radish	<i>Raphanus raphanistrum</i>	Brassicaceae	Europe, Asia
		Rhododendron	<i>Rhododendron</i> spp.	Ericaceae	Temperate N hemisphere
	⊕	Rose	<i>Rosa hybrida</i>	Rosaceae	N temperate
97	**	Raspberry	<i>Rubus idaeus</i>	Rosaceae	Europe, Asia, N America
osd	*	Sugarcane	<i>Saccharum officinarum</i>	Gramineae	Tropics, spreading N & S with interspecific hybridisation
		African violet	<i>Saintpaulia ionantha</i>	Gesneriaceae	Coastal Tanzania
		Clary	<i>Salvia sclarea</i>	Labiatae	S Europe
		White mustard	<i>Sinapis alba</i>	Brassicaceae	Mediterranean littoral, especially Aegean
96,osd		Eggplant / brinjal / aubergine	<i>Solanum melongena</i>	Solanaceae	India
		Black nightshade	<i>Solanum nigrum</i>	Solanaceae	Europe
16,osd		Potato	<i>Solanum tuberosum</i>	Solanaceae	Tropical highlands of S America
56		Sorghum	<i>Sorghum bicolor</i>	Gramineae	Semi-arid tropical East Africa
	*	St. Augustine grass	<i>Stenotaphrum secundatum</i>	Gramineae	Warm Americas
	⊕	African/Cape marigold	<i>Tagetes erecta</i>	Asteraceae	C America
		Torenia	<i>Tourenia fournieri</i>	Scrophulariaceae	Vietnam
		Hare's foot clover	<i>Trifolium arvense</i>	Leguminosae	Europe
24	**	White clover	<i>Trifolium repens</i>	Leguminosae	Europe, Asia, N Africa
24	**	Subterranean clover	<i>Trifolium subterraneum</i>	Leguminosae	W Europe, Asia minor, N Africa
1,osd	**	Wheat	<i>Triticum aestivum</i>	Gramineae	only cultivated
11	*	Wheat, durum	<i>Triticum turgidum</i> ssp. <i>durum</i>	Gramineae	only cultivated
		American elm	<i>Ulmus americana</i>	Ulmaceae	E N America
		Blueberry	<i>Vaccinium</i> spp.	Ericaceae	
		Cranberry	<i>Vaccinium</i> spp.	Ericaceae	
		Adzuki bean	<i>Vigna angularis</i>	Leguminosae	NE Asia
		Grape	<i>Vitis berlandieri</i> X <i>V. riparia</i>	Vitaceae	N America
		Grape	<i>Vitis berlandieri</i> X <i>V. rupestris</i>	Vitaceae	N America
		Grape - sand grape	<i>Vitis rupestris</i>	Vitaceae	N America
18,54, osd	**	Grape	<i>Vitis vinifera</i>	Vitaceae	Wild <i>V. vinifera</i> C Asia, Afghanistan to Black & Caspian Seas

Table 2.10 *continued*

Rank	Risk	Crop	Family	Native to
		Grape	<i>Vitis vinifera</i> X <i>V. berlandieri</i>	Vitaceae
5,8,osd		Corn/maize	<i>Zea mays</i>	Gramineae
		Zoysiagrass	<i>Zoysia matrella/japonica</i>	Gramineae
				Tropical Asia
		Definitions:		
	osd	cultivated in overseas departments of Europe		
	fs	European forestry species		
	grey text	not cultivated in Europe		
	plain text & background	cultivated in Europe with no potential for gene flow		
	**	hybrids recorded in Europe		
	*	related species native/naturalised in Europe		
	⊕	ornamentals with potential for gene flow		

2.3.2 Major crop species

Of the 20 top-ranking crops (Table 2.6), eight pose some risk of transgene escape via hybridisation. Europe's top crop species, wheat (*Triticum aestivum*) is known to hybridise with spelt wheat (*T. spelta*), which occurs in central, and NW Europe (Smartt & Simmonds 1995; Jørgensen & Wilkinson 2005). In addition, *T. aestivum* will hybridise with several species of *Aegilops* (*A. speltoides*, *A. ventricosa*, *A. cylindrica*, *A. triuncialis* and *A. geniculata*) (Tutin *et al.* 1964-1980; Ellstrand 2003b Jørgensen & Wilkinson 2005) all of which occur in SE Europe (Smartt & Simmonds 1995).

Barley (*Hordeum vulgare*) is the second most widely grown crop species in Europe and ranks third in area after wheat and permanent pasture (Table 2.6). It is known to hybridise freely with wild *H. spontaneum* and *H. distichon* (Tutin *et al.* 1964-1980; Jørgensen & Wilkinson 2005). *H. spontaneum* occurs in dry places in Crete and western parts of Asia and *H. distichon* is cultivated as a minor cereal crop in most parts of Europe (Tutin *et al.* 1964-1980; Stace 1975; Smartt & Simmonds 1995).

Maize appears to pose little risk of hybridisation with wild species in Europe and is cultivated widely both as a grain and forage crop, ranking fifth and eighth in the list respectively. Oilseed rape (*Brassica napus*), ranking ninth, is of much greater concern, as it will hybridise with several other *Brassica* species that are native to Europe, along with *Raphanus raphanistrum* and *Hirschfieldia incana* (Tutin *et al.*

1964-1980; Smartt & Simmonds 1995; Ellstrand 2003c; Jørgensen & Wilkinson 2005).

The fifth ranking crop species, durum wheat (*Triticum turgidum* ssp. *durum*) will hybridise with *Aegilops geniculata*, *A. neglecta* and *A. ventricosa*. These species occur across southern parts of Europe (Tutin *et al.* 1964-1980; Smartt & Simmonds 1995; Ellstrand 2003c; Fehr & Hadley 2003).

Sunflowers (*Helianthus annuus*), ranked twelfth in terms of area, are native to North America, but have escaped cultivation in Europe and are locally naturalised in the wild. Hybridisation between sunflowers has been recorded at a long distances and may offer a route for gene escape from a transgenic crop (Jørgensen & Wilkinson 2005). In addition, *H. annuus* can cross with *H. tuberosus* (Jerusalem artichoke), which is also cultivated in Europe (Ladizinsky and Zohary 1971; Stace 1975; Ellstrand 2003c; Jørgensen & Wilkinson 2005).

Oats (*Avena sativa*) rank as the thirteenth most highly cultivated crop (Table 2.6) and are inter-fertile with three other wild and cultivated species of *Avena* that are common in Europe (Jørgensen & Wilkinson 2005). The hybrids formed are highly fertile (Ladizinsky & Zohary 1971). *A. sativa* itself is an aggregate of wild, weedy and cultivated forms and is widespread throughout most of Europe, representing further opportunities for transgene escape (Tutin *et al.* 1964-1980; Ellstrand 2003c).

Potatoes are the sixteenth highest-ranking crop species and are native to South America. As such, they represent a lesser risk of transgene escape as none of the species known to hybridise with *Solanum tuberosum* are recorded in Europe (Tutin *et al.* 1964-1980; Fehr & Hadley 1980; Ellstrand 2003c).

Sugar beet (ranked seventeenth) and all other cultivated beets, including fodder beet (45th) and beetroot (92nd), belong to the same subspecies, *Beta vulgaris* subsp. *vulgaris*. All will hybridise with the wild subspecies *B. vulgaris* subsp. *maritima* which occurs in coastal areas of Europe and occasionally inland as an arable weed. In addition, cultivated beets will also hybridise with several other *Beta* species, but only *B. macrocarpa* is present in Europe (Smartt & Simmonds 1995; Ellstrand 2001).

Vitis vinifera occurs at number 18 in the ranking as 'wine grapes' and at number 54 as 'table grapes' (Table 2.10). The wild progenitor species of cultivated grapes is *V. sylvestris*, which occurs across southern Europe. This species will readily cross with cultivated vines (Tutin *et al.* 1964-1980; Smartt & Simmonds 1995; Ellstrand 2001; Jørgensen & Wilkinson 2005) and spontaneous crossing between the two species is common where the two are sympatric. The taxonomy of the group is now difficult due to the presence of a complex of wild, weedy, cultivated and escaped varieties in the Mediterranean area. F₁ hybrids are fully fertile, thus allowing introgression of genes between species and making species delimitation difficult (Tutin *et al.* 1964-1980). Additionally, several American wine grape species have now been introduced into Europe and these are becoming naturalised in some areas (Smartt & Simmonds 1995). These species are also capable of crossing with *V. vinifera* to form fertile hybrids (Smartt & Simmonds 1995).

The most recent relevant information of field trials of GM grapes (Vigne *et al.* 2004) comes from the announcement that after a six-year suspension, researchers at the National Institute for Agricultural Research (INRA) in Colmar in the Alsace region of France resumed field experiments in the beginning of September 2005 (Vermij 2005). The GM rootstocks are engineered to resist infection by the devastating grapevine fanleaf nepovirus. Non-GM scions of *Pinot Meunier*, a grape not otherwise used for wine making in the Alsace region, will be grafted on top of the rootstocks. Floral buds will be cut, so no GM grapes (or wine) will be produced.

2.3.3 Forage and fodder crops

Data for individual forage species other than lucerne/alfalfa in Europe have not been obtained and thus the extent of cultivation of specific forage species can only be assumed from data obtained on the area under fodder and pasture crops (Table 2.6). As these rank highly (perennial green fodder 6, annual green fodder 7) it can be assumed that fodder species are cultivated extensively in Europe. In comparison to arable crop species, there are relatively few fodder crops for which GM field trial applications have been made and therefore the number of species to be considered

is relatively low. Varieties of maize and beet are cultivated as fodder crops, but as these share the same species delimitations as those cultivated for human consumption they are discussed elsewhere (section 2.3.1, 2.3.2).

Forage crops for which field trial applications have been made are alfalfa/lucerne (*Medicago sativa*), barrel clover (*M. truncatula*), white clover (*Trifolium repens*), subterranean clover (*T. subterraneum*) and the narrow-leaved lupin (*Lupinus angustifolius*) (Table 2.2).

2.3.3.1 *Medicago*

Medicago sativa is listed as an individual species by Eurostat and it ranks nineteenth in terms of acreage (Table 2.6). In the wild, *M. sativa* is a complex of three subspecies that readily hybridise, but occur in slightly different habitats. *M. sativa* ssp. *falcata* prefers cooler climates and occurs in northern Europe and extends as far as Siberia. *M. sativa* ssp. *sativa* and ssp. *falcata* occur in the milder climates of the Mediterranean, extending to the Caucasus and central and south Asia. These two subspecies hybridise where sympatric (Stace 1975; Smartt & Simmonds 1995). In addition, the two closely related species *M. glomerata* in southern Europe, and *M. falcata* widespread throughout Europe, will both hybridise freely with *M. sativa* (Tutin *et al.* 1964-1980).

Little hybridisation information has been found on the less widely cultivated *M. truncatula* (a model legume for genomic research), although the species is recorded wild from many of the Mediterranean states and islands (Tutin *et al.* 1964-1980).

The majority of field trials of alfalfa involve herbicide tolerance traits. For example, in the US, Monsanto has made 86% of trial applications for alfalfa and these varieties carry tolerance to glyphosate. Indeed, Monsanto have a deregulated Roundup-ready alfalfa variety that is commercially available in the US (<http://www.isb.vt.edu/cfdocs>).

Trials have also been conducted on alfalfa for stress tolerance and altered biosynthesis pathways in the US and Canada. Field trial applications for GM alfalfa have also been made in Argentina, Belgium, Bulgaria, Mexico and Spain (Table 2.2).

2.3.3.2 *Trifolium*

Of the two species of *Trifolium* for which GM varieties are being developed, more is known about the hybridisation potential of *T. repens* than *T. subterraneum*. Nonetheless, both species occur wild throughout most of Europe (Tutin *et al.* 1964-1980). *T. repens* will hybridise with fifteen other species of *Trifolium* in Europe, of which five are endemic to certain areas. *T. repens* itself is a complex of six subspecies, of which four are endemic (Smartt & Simmonds 1995).

Field trials of *Trifolium* species have been conducted in Australia, Canada and New Zealand (Table 2.2). These trials have been testing varieties with resistance to herbicide, viral disease and stress.

A small number of trials of transgenic *Trifolium arvense* have been conducted in New Zealand (Table 2.2). This species occurs across Europe and the UK but it is not widely cultivated as a forage species (Smart & Simmonds 1995). The hybridisation potential of this species has therefore not been considered here.

2.3.3.3 Lupin

Hybridisation of cultivated lupins is a recognised problem as species are highly inter-fertile and 'sweet' varieties cultivated for fodder are known to become bitter due to hybridisation with sympatric wild types (Tutin *et al.* 1964-1980). The European flora contains ten species of lupin, of which six are native annual species, including *Lupinus angustifolius* (Table 2.2), and four are introduced American perennials (Tutin *et al.* 1964-1980; Stace 1975).

Lupins have been field trialled in Australia (Table 2.2) for traits including altered seed composition and sulphur levels.

2.3.3.4 Summary of forage and fodder crops

These data show that for all the potential GM forage crops that may be cultivated in Europe, transgene escape to wild and related wild species is probable, with the exception of green fodder maize.

2.3.4 Forestry Species

Unlike arable crop species, forestry species are much longer lived and are often farmed from natural/semi-natural woodland as well as from planted forest. The species utilised are thus not improved varieties as for conventional crop species. As such, forestry species are identical to those species growing in the wild and thus, hybridisation with wild populations will have no genetic barrier and is of primary concern.

The increasing number of applications for field-testing of GM forest trees in the US is shown in Figure 2.2. Of the 20 major forest species in Europe (Table 2.9), six have been included in GM field trials, of which four involve native species present across Europe. However, details of the exact species transformed are not given in some

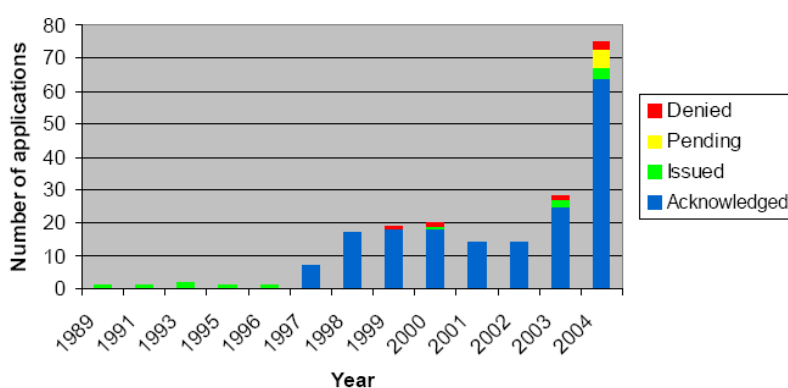


Figure 2.2 Field test applications for GM forest trees in the US

(Source: APHIS data from FAO 2004)

applications (i.e. *Betula* spp.) so an exact number is not possible at this point. The generic field trials applications name *Betula* spp., *Picea* spp., *Pinus* spp., *Populus* spp. and *Ulmus* spp. all of which have species native in Europe. Development of GM varieties and hybridisation between species of *Betula*, *Picea*, *Pinus* and *Populus* are discussed below.

Commercial, and academic, interest in transgenic tree breeding (Walter and Killerby 2003; Boerjan 2005) is based on efforts to increase tree productivity, improve manufacturing efficiency and product quality, and introduce disease resistance and control over reproductive traits (Cooke *et al.* 2004; Powell *et al.* 2005). There is also an emerging interest in the use of GM trees to generate bio-pharma products and for phytoremediation (Peuke & Rennenberg 2005).

Insect resistance has been engineered into trees species by the insertion of the Bt insecticidal toxin genes in a wide range of tree species including poplar (McCown *et al.* 1991), eucalyptus (Harcourt *et al.* 2000), larch (Shin *et al.* 1994), and spruce (Peña & Séguin 2001). Alternative approaches to confer insect resistance have been developed; these include the over-expression of proteinase inhibitors that interfere with digestive processes in herbivorous insects (Leplé *et al.* 1995; Delledonne *et al.* 2001).

There are arguments that suggest the genetic modification of native and landscape trees may be beneficial in a conservational context, since introduced pests and diseases have devastated populations of many native tree species of the North Temperate Zone. For example, populations of the English elm (*Ulmus procera*) have been decimated by *Ophiostoma ulmi*, Dutch elm disease. As trees have such an extended life span, generating disease resistant lines by conventional methods may well be insufficient. Adams *et al.* (2002) argue that limited transfer of resistant genes from the original host species may enable many North Temperate tree species to retain and resume their ecological niche.

2.3.4.1 Poplar

Of all forest tree species most transgenic research has been conducted on poplar (Marchadier & Sigaud 2005) (Figure 2.3) with the majority of this research concentrated in the USA (Figure 2.4) (see also www.fao.org/docrep/008/ae574e/ae574e00.htm). *Populus* is a good model species for genetic studies in trees as it grows rapidly, can be vegetatively propagated, is amenable to tissue culture and to transformation protocols and it has a relatively small genome size (Cooke *et al.* 2004). The *Populus* genus includes species that are native in Europe and are

reasonably widespread. In addition, there are species that have been introduced from North America and Asia. Both the native and the introduced species will

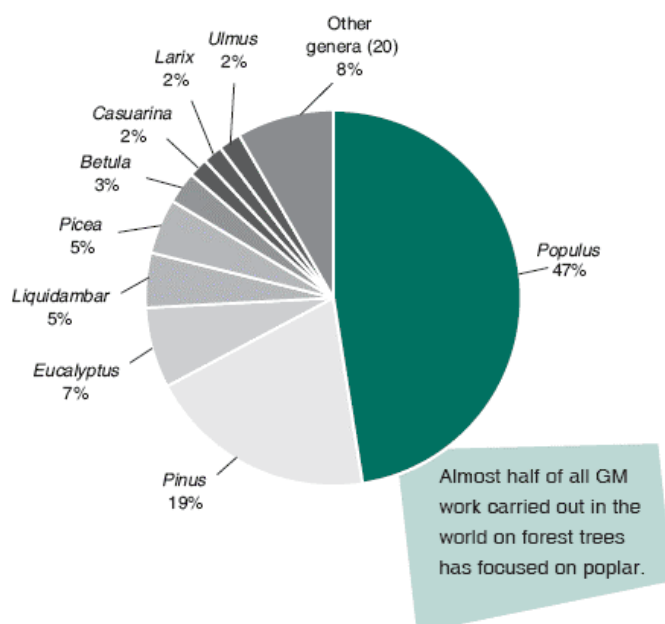


Figure 2.3 Transgenic research on trees according to genus
(Source: Marchadier and Sigaud 2005)

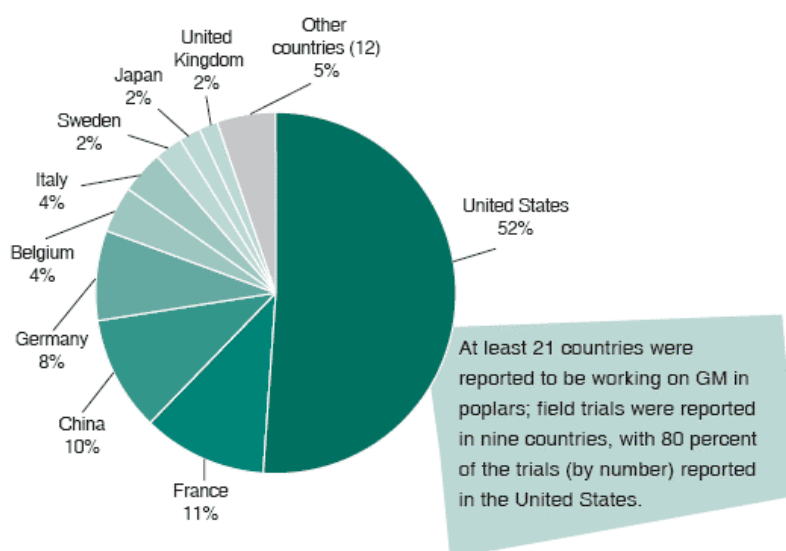


Figure 2.4 Transgenic research on poplar according to country
(Source: Marchadier and Sigaud 2005)

hybridise and these hybrids are sufficiently common to have been given specific names. Poplar trees, including transgenic specimens, are also capable of vegetative

propagation offering a means of reproduction without pollination (Tutin *et al.* 1964-1980; Stace 1975).

The predominant focus of this research has been herbicide tolerance (Figure 2.5). Herbicides are used in intensive forestry management to reduce competition from weedy species and thus the development of herbicide-resistant trees would be advantageous to the industry. Resistance to glyphosate has been engineered into poplars (Donahue *et al.* 1994; Meilan *et al.* 2002) and into larch (Shin *et al.* 1994), and resistance to glufosinate ammonium into poplar and eucalyptus (Confalonieri *et al.* 2000; Harcourt *et al.* 2000).

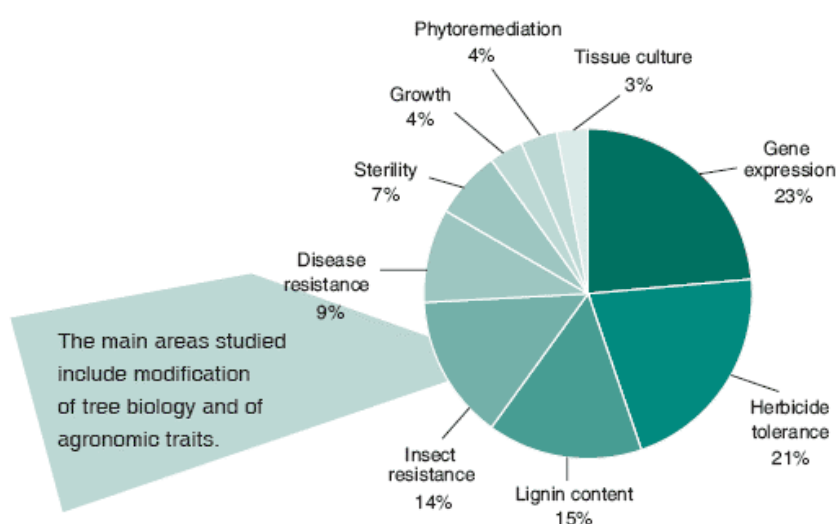


Figure 2.5 Transgenic research on poplar according to gene of interest
(Source: Marchadier and Sigaud 2005)

Improvements to wood quality and a reduction in the environmental impact of the pulping process may be attained through the genetic modification of the lignin biosynthesis pathway. This pathway is well documented and has been the subject of much investigation in the past due to its importance in wood and paper quality. Thus more progress has been made on manipulating lignin than any other trait affecting wood quality (Cooke *et al.* 2004). However, modification of lignin can result in aberrant tree growth and may have negative effects on fibre characteristics and indeed, may render the wood more susceptible to infection from fungal pathogens and disease (Vance *et al.* 1980; Legrand *et al.* 2000).

Several strategies have been used in poplar to confer general disease resistance including expression of oxalate oxidase from wheat (Liang *et al.* 2001), introduction of a bacterio-opsin gene from *Halobacterium halobium* (Mohamed *et al.* 2001), and expression of synthetic antimicrobial peptides that cause pathogen mortality (Liang *et al.* 2002). Engineering resistance to specific diseases is also being explored. For example, resistance against the fungal rust *Melampsora* has been ascribed to a single dominant gene in *Populus* spp. (Newcombe *et al.* 1996) and diminished crown gall formation by *Agrobacterium tumefaciens* was achieved by transformation of *Populus* spp. with antisense *iaaM* (Ebinuba *et al.* 1997).

As a consequence of this emphasis on *Populus* species in transgenic tree research (Fladung *et al.* 2003; 2004; Kumar and Fladung 2004) they represent the only GM trees that are grown extensively under non-restricted conditions, albeit only in China to date. Growth of GM poplars in China has been ongoing since 1993 (Tian 1998, Wang 2004) and is continuing. An insect resistant line of *P. nigra* expressing Bt ('Poplar-12') is being grown over 200 ha of the northern regions of China including Hebei, Beijing, Liaoning and Ningxia. Another commercialised transgenic species, 'Poplar-741', expressing Bt plus another enzyme, is growing in sporadic plantations totalling less than 3 ha across nine provinces and municipalities including Hebei, Beijing, Tianjin and Shandong. Both lines are reported to be female poplars with altered fertility, and are unable to release pollen (China Daily, 1st April 2005, <http://www.china.org.cn/english/environment/124471.htm>).

Recent related studies on inducing improved salinity tolerance have included tests on the utility of mannitol-1-phosphate dehydrogenase, which catalyzes the biosynthesis of mannitol from fructose. The gene expressing this enzyme (*mtID*) was cloned from *E. coli* and transferred to poplar (*Populus tomentosa*) through *Agrobacterium*-mediated transformation (Hu *et al.* 2005).

Wang (2004) reports that in May 2003, the GM poplar expressing this gene was officially registered for the protection of breeder's right under a Chinese common name, Taiqing No.1 poplar (PVP Office 2003; <http://www.cnvp.net/announcement1.htm>). In June 2003, the environmental release was officially approved by the Ministry of Agriculture to establish 100 mu (equal to about 6.6 ha) of

plantations in Tianjin City and Shandong Province. In December 2003, the research project was thoroughly reviewed; the GM poplar was given another Chinese common name, Zhongtianyang, and assigned a cultivar name as (*Populus* × *xiaozhuanica* W.Y. Hsu and Liang cv. 'Balizhuangyang-zhongtian'). This GM line has now established in coastal areas in Shandong Province, where the soil has been so seriously saline that there is a limited choice of tree species for planting (Wang 2004). Table 2.11 provides a summary of the status of GM tree research in China.

Table 2.11 Summary of genetic modification in forest trees in China.

R: research; E: environmental release; C: commercial planting. (Source: Wang 2004)

Species	Status	Traits	Gene	Development
<i>Betula platyphylla</i>	R	Insect resistance	Spider insecticidal peptide	On going
<i>Eucalyptus camaldulensis</i>	R	Lowering lignin content	C4H	On going
<i>Eucalyptus urophylla</i>	R	Resistance to disease caused by <i>Pseudomonas solanacearum</i>	cecropin D	On going
Poplar hybrid 741 (<i>P. alba</i> × [<i>P. davidiana</i> + <i>P. simonii</i>] × <i>P. tomentosa</i>)	E, C	Resistance to leaf-eating insects	<i>Bt Cry1</i> and API	Applied for commercial plantings in 2001
<i>Populus</i> × <i>xiaozhuanica</i> W.Y. Hsu et Liang cv. 'Balizhuangyang-zhongtian'	E	Salt tolerance	<i>mtlD</i>	Approved for environment release in 2003. Granted breeder's right in 2003
<i>Populus deltoides</i>	R	Insect resistance	<i>Bt</i>	On going
<i>Populus deltoides</i> × <i>P. cathayana</i>	R	Resistance to leaf-eating insects	<i>mtlD/gutD</i>	On going
<i>Populus deltoides</i> × <i>P. simonii</i> (N-106)	R	Resistance to leaf-eating insects	<i>AaIT</i>	On going
<i>Populus nigra</i>	E, C	Resistance to leaf-eating insects	<i>Bt</i>	Applied for commercial plantings in 2002
<i>Populus simonii</i> × <i>P. nigra</i>	R	Salt tolerance.	<i>Bet-A</i>	On going
<i>Populus tomentosa</i>	R	Resistance to disease and stress	<i>NP-1</i> (rabbit alexin)	On going

Transgenic varieties of *Populus* are also being developed in the USA, though not yet on a commercial basis. Oregon State University laboratory has generated more than 6,500 independent gene-transfer events in 17 different genotypes of *Populus*, and carried out field-tests of more than 1,600 of these lines (Brunner *et al.* 2004). This work has focussed on tree maturation, flowering and stature. This research has generated stable gene expression system for vegetatively propagated transgenic poplars with manageable amounts of somaclonal variation. The values of transgenic traits are reported to be high, but broader evaluation is required to determine commercial potential. In addition, sterility has been induced via a range of methods (Brunner *et al.* 2004).

Field trials of GM poplar varieties have also been conducted in Belgium, Canada, France, Germany, Norway, Spain (Jing *et al.* 2004), Sweden and the UK (Table 2.2).

2.3.4.2 Birch

The silver birch (*Betula pendula*) is native across most of Europe and will hybridise with the other widespread species *B. pubescens*, and with *B. nana*, which occurs mainly in the northern European states (Tutin *et al.* 1964-1980). The silver birch is an economically important species in the northern states of Europe and is one of the key species in Nordic forest ecosystems. It constitutes approximately 15% of the growing stock of Finland and has been the focus of conventional breeding of broad-leaf tree species (Aronen *et al.* 2004).

Genetic modification of silver birch is less advanced than that of poplar and considerably fewer field trials of GM varieties have been conducted (Table 2.2). However, several groups are developing new transgenic lines. Resistance to fungal pathogens has been induced by the introduction of a chitinase gene from sugar beet (Pappinen *et al.* 2002) and some of these lines have been field trialled in southern Finland (Pasonen *et al.* 2004). Flowering control is also being investigated by research into the genes controlling flower formation (Lemmetyinen *et al.* 2004a; b). Research is also progressing into the genetic modification of lignin biosynthesis to generate transgenic lines that will reduce the ecological impact of pulp and paper manufacture (Aronen *et al.* 2004).

Field trials of GM birch have been conducted in China and Finland (Table 2.2)

2.3.4.3 Chestnut

Sweet chestnut (*Castanea sativa*) is native to parts of south and central Europe and has been introduced to many other countries. There is only one other European species of *Castanea* (*C. crenata*), an introduced timber species, but there are no records of hybridisation between these two species (Tutin *et al.* 1964-1980).

Chestnuts have economical importance because of their fruits and high quality timber and play an important ecological and landscape function. They are prone to fungal attack and numbers of chestnut trees have suffered severely over the past 100 years (Seabra & Pais 1998). Indeed, chestnut reforestation has been carried out using hybrids of *C. sativa* x *C. crenata* as they have greater resistance to the fungal pathogen. However, the hybrids produce wood of inferior quality and numbers of *C. sativa* are rapidly declining in countries such as Portugal, where research is developing transgenic *C. sativa* clones with the aim of introducing disease resistant traits (Seabra & Pais 1998). A small number of field trials of transgenic American chestnut (*C. dentata*) (Polin *et al.* 2005) have been carried out by New York State University and a single application has been made for a trial of GM *C. sativa* in France (Table 2.2).

2.3.4.4 Spruce

The Norway spruce (*Picea abies*) is native to the mountains of northern Greece and Bulgaria and has been introduced into much of the rest of Europe as a timber species. The only other native *Picea* species in Europe is *P. omorika*, which is cultivated in some countries for timber, but only occurs wild in the Drina basin in Bosnia-Herzegovina and Yugoslavia. Other *Picea* species have been introduced into Europe for timber, mostly from North America, but there are no records of hybridisation between species recorded (Tutin *et al.* 1964-1980; Stace 1975).

Transformation of *Picea abies* has focussed on the introduction of herbicide resistance (Brukhin *et al.* 2000; Bishop-Hurley *et al.* 2001). More recent research has been investigating the control of pollen and seed development and the transition from juvenile, non-reproductive growth into adult growth (Carlsbecker *et al.* 2003; 2004). Trials of GM *Picea* have been conducted in Finland, New Zealand and the USA (Table 2.2).

2.3.4.5 Pine

Pinus sylvestris is widespread across the uplands and mountains of Europe. The three other *Pinus* species native to Europe are endemic in the mountains of the

Balkans and the Alps, Pyrenees and Carpathians. Eight other *Pinus* species have been introduced as timber species from North America, west Asia and one from the Canary Islands. One of the introduced species is Monterey pine (*Pinus radiata*), for which GM lines have been developed and field trial applications made (Table 2.2). As these eight species have geographically disparate origins to *P. sylvestris*, it is possible that there will be no genetic barriers to hybridisation. There are currently no records of hybridisation events between the native and introduced species (Tutin *et al.* 1964-1980) but the absence of data does not necessarily imply the absence of crossing.

Development of GM varieties of pine is second only to poplar, with *P. radiata* the most commonly transformed species of pine (van Frankenhuyzen & Beardmore 2004). Traits engineered into pine include glufosinate resistance (Bishop-Hurley *et al.* 2001), disease resistance (Séguin 1999), salt tolerance (Tang 2002) and induced sterility (Mouradov *et al.* 1998). Trials of some of these lines have been conducted in Finland, New Zealand and the USA (Table 2.2).

2.3.4.6 *Eucalyptus*

One of the introduced timber species that has reached GM field trials is Tasmanian blue gum (*Eucalyptus globulus*). There are no native species of *Eucalyptus* present in Europe, although many have been introduced as timber species. As yet, there are no details of hybridisation events between these species in Europe (Tutin *et al.* 1964-1980; Stace 1975) but spontaneous hybridisation between eucalypt species has been noted in parts of Australia (Barbour *et al.* 2003; 2005).

Eucalyptus species have been engineered to confer tolerance to glyphosate (Llewellyn 2000) and to insect infestation (Harcourt *et al.* 2000). In addition, field trials have been carried out in the US of eucalypts with altered lignin biosynthesis pathways (<http://www.isb.vt.edu/cfdocs/>) and recently (1 Nov 2005) it was announced that Nippon Paper Industries (NPI) has started an outdoor cultivation experiment with salt tolerant eucalypts in collaboration with the University of Tsukuba. These trees express a choline oxidase gene from *Arthrobacter globiformis*, and their salt tolerance has been confirmed in the previous greenhouse experiment

in which the trees were supplied with saltwater of 30% salinity, the same salinity as seawater. Additional field trial applications for GM eucalypts have been made in Brazil, Portugal, South Africa, Spain and the UK (Table 2.2).

2.3.4.7 Elm

Although there have been no field trials of genetically modified English Elm (*Ulmus procera*) there are two applications and one pending from New York State University to grow genetically modified American elm (*Ulmus americana*). The trees have been engineered to express synthetic magainin and cecropin genes (Figure 2.6; US Patent 5856127) as a means to improve tolerance to Dutch elm disease. These field trials began in 2005. Transformation of *Ulmus procera*, and regeneration of transformants has been demonstrated by research at the University of Abertay, Dundee (Gartland *et al.* 2000; 2001). Should the American trials prove successful, the development of English elm resistant to Dutch elm disease should also be possible.



Figure 2.6 Transgenic American elm
(Source: <http://www.esf.edu/efb/powell/research.html>)

2.3.4.8 Oak

The recent problem of sudden oak death (*Phytophthora ramorum*) has also generated renewed interest in protection against this disease. The pathogen itself is the subject of a genome sequencing project funded by the US Department of Energy (http://genome.jgi-psf.org/euk_cur1.html), and although there have been no reported trials of transgenic oak, there are laboratory studies of the cork oak *Quercus suber* (Álvarez *et al.* 2004) and the sawtooth oak *Q. acutissima* (Taniguchi *et al.* 2003).

2.3.4.9 Summary of forestry species

All timber species for which GM field trial applications have been made have the potential to hybridise with tree species that occur in the wild, with the exception of *Eucalyptus globulus*, the Tasmanian blue gum. However, the cross-compatibility relationships between the blue gum and the other *Eucalyptus* species present in Europe requires further investigation before the risk of transgene spread can be completely ruled out. In addition, an initiative launched last year in the US to improve hardwood quality via genetics will undoubtedly increase the number species for which GM technology has been developed (Merkle & Nairn 2005). A new National Science Foundation research centre dedicated to improving the quality of valuable hardwood tree species will be lead by Purdue University. This will advance work in improving the quality of hardwood tree species native to Indiana and much of the Midwest, such as black walnut, northern red oak and black cherry, which are highly prized by the fine furniture and cabinetry industries.

2.3.5 Orchard crops

Small numbers of field trials have been conducted on a variety of fruit trees and orchard crops that are cultivated in Europe (Table 2.2, 2.6).

2.3.5.1 Olives

Olives are the highest-ranking orchard crops in Europe in terms of area (Table 2.6). The cultivated olive (*Olea europea*) is native to the Mediterranean basin and often occurs with its wild progenitor species *O. sylvestris* (Tutin *et al.* 1964-1980). The two species are fully interfertile and sporadic hybridisation occurs where they are in proximity (Tutin *et al.* 1964-1980; Smartt & Simmonds 1995). Cultivated olive trees also occur as feral populations (Ellstrand 2003c).

To date there have been two field trials of GM olives conducted in Italy by Università degli Studi della Tuscia (Table 2.2).

2.3.5.2 Apples

Apples rank 26 in terms of acreage in Europe (Table 2.6) and they are present in most European states under cultivation and as escapes (Tutin *et al.* 1964-1980). The cultivated apple will readily hybridise with most species of *Malus* (Smartt & Simmonds 1995) of which there are six present in Europe, including one endemic species in the Balkan States and Italy (Tutin *et al.* 1964-1980).

To date, there have been 48 field trial applications for GM apples in Argentina, Belgium, Germany, The Netherlands, New Zealand, Sweden, UK and USA (Table 2.2).

2.3.5.3 Plums and cherries

Plums and cherries are less widely cultivated in Europe, ranking 40 and 42 in terms of acreage (Table 2.6). The cultivated plum (*Prunus domestica*) and the sweet cherry (*P. avium*) will cross with many of the other *Prunus* species occurring in Europe including wild types, those cultivated for timber and ornament, as well as the other fruit-bearing *Prunus* species (peach, almond, damson, greengage) (Stace 1975; Smartt & Simmonds 1995). In all there are 21 species of *Prunus* in Europe, including one species endemic to southern Spain (Tutin *et al.* 1964-1980).

Despite plums being a lower acreage crops than olives and apples, the development of GM lines of plums is further developed. To date there have been 10 field trial applications for GM plums in the US, Italy and Spain (Table 2.2). In addition ARS currently have a petition pending in the US for the deregulation of a GM line of plum resistant to plum pox virus (Appendix VI). There have been fewer trials of GM cherries, with only three applications being made in Italy (Table 2.2).

2.3.5.4 Pear

Pears (*Pyrus communis*) are native to Asia Minor and the Caucasus and rank 53 as a European crop (Table 2.6). Most cultivated pears are able to cross with other *Pyrus* species and varieties (Smartt & Simmonds 1995) and possibly with *Sorbus aria* (whitebeam) (Stace 1975). There are 13 species of *Pyrus* in Europe, the majority of which are endemic (Tutin *et al.* 1964-1980) and includes one of Britain's rarest trees, the Plymouth pear (*Pyrus cordata*).

Development of GM lines of pear have so far been limited with only six field trial applications having been made in Sweden and the US (Table 2.2).

2.3.5.5 Walnut

Walnuts (*Juglans regia*) rank 83 in terms of acreage in Europe (Table 2.6) and are native to the area extending across the southeastern states of Europe (Greece and the Balkan Peninsula) to China. There are three species of *Juglans* present in

Europe of which only *J. regia* is native. The other species have been introduced for fruit and timber production and have become naturalised (Tutin *et al.* 1964-1980). Many *Juglans* species are interfertile and it is likely that these species will be cross-compatible with each other (Simmonds 1976).

To date there have been 15 field trial applications for lines of GM walnut, all of which have been conducted in the US (Table 2.2).

2.3.5.6 *Citrus*

There are nine species of *Citrus* present in Europe all of which have been introduced and are cultivated on different scales for their fruit and essential oils (lemon, lime, sweet orange, Seville orange, mandarin, grapefruit, bergamot, pomelo/ugli fruit and citron) (Tutin *et al.* 1964-1980). Of these species, oranges are the most widely cultivated in terms of area, ranking 51, followed by lemon (86), grapefruit (121) and lime (133). *Citrus* species are native to Southeast Asia and are widely interfertile between species. Hybrids of interspecific crosses are also often fertile and this has been exploited by breeders to generate a wide variety of citrus fruit forms (Smartt & Simmonds 1995).

There have been relatively few GM field trial applications for *Citrus* trees (lime, 1; lemon, 2; orange, 1; grapefruit, 6) based in the US, Mexico, Italy and Spain (Table 2.2) demonstrating the limited development of GM technology in this group of crops.

2.3.5.7 *Avocado*

Avocados (*Persea americana*) have been introduced into Europe from the Americas and they are cultivated on a relatively small scale in parts of France and Cyprus, ranking only 125 in terms of area (Table 2.6). The three cultivated races of avocado constitute subspecies of *P. americana* and these are all cross-compatible with each other (Smartt & Simmonds 1995). *P. americana* does not appear to have escaped cultivation and does not exist in feral populations. However, there is one species of

Persea (*P. indica*) native to some parts of Europe. This species is native to Madeira and to the Canary Islands and has been introduced and become naturalised in the Azores (Tutin *et al.* 1964-1980). It is as yet unclear whether *P. americana* and *P. indica* are cross-compatible with each other.

There has been very little development of GM lines of avocado with only one field trial application having been made to date in the US (Table 2.2).

2.3.5.8 Papaya

Papaya is cultivated on a very small scale in Europe, with data suggesting cultivation only occurs in Madeira (Table 2.7). The species is native to tropical America and as such there are no related species in the European flora (Tutin *et al.* 1964-1980). The development of GM lines of papaya is relatively well developed, however, with one variety of virus-resistant GM papaya that has been deregulated by APHIS (Appendix V) and a second variety with an application for deregulation currently pending (Appendix VI). These two varieties have been developed in the US by Cornell University, the University of Hawaii and the University of Florida, and represent the only GM fresh fruit on the market in the world. In addition to field trials in the States, field trial applications for GM papaya have also been made in Australia, Brazil, China, Cuba, Japan and Mexico (Table 2.2).

2.3.5.9 Summary of orchard crops

The majority of orchard crops planted in Europe pose some risk of transgene escape to wild relatives with olives, apples, plums, cherries, pears and walnuts all native to Europe or the Mediterranean area and all possessing cross-compatibility with related species. Development of GM lines of these crops is not advancing rapidly, however, with only small numbers of field trial applications having been made. The most developed GM variety is a plum pox resistant plum tree developed by ARS in the US currently with an application for deregulation currently pending.

The remaining fruit trees (*Citrus*, avocado, papaya) are all introduced to Europe and are cultivated on very small scales. As these trees have no wild relatives in the

European flora, the risk of transgene spread is thus restricted to crop-to-crop pollination. There are two virus-resistant lines of GM papaya, one which has been deregulated in the US and one with an application for deregulation pending. These are the only GM fruit grown commercially in the world. GM lines of *Citrus* and avocado are not well developed.

2.3.6 Grasses

As in the case with tree species, grasses cultivated for forage or amenity are often very similar to those species growing in the wild. Of the 13 species of grass for which GM field trial applications have been made, ten occur in Europe. Grasses have a very effective self-incompatibility mechanism that makes the group inherently out-crossing. Many grasses are cross-compatible with species in the same genus, and in some cases in other genera.

2.3.6.1 *Agrostis*

GM field trials of two species of *Agrostis* (*A. canina* and *A. stolonifera*), have been conducted (Table 2.2). These species will hybridise with each other and both occur across most of Europe (Tutin *et al.* 1964-1980). In addition, *A. stolonifera* is known to cross with at least two other species in the genus. All 25 species within the genus in Europe are believed to have the potential to hybridise with each other (Tutin *et al.* 1964-1980).

Only one field trial of GM *A. canina* has been conducted (Table 2.2). This trial of a phosphinothricin tolerant cultivar took place in the US by the University of Rhode Island. *A. stolonifera* conversely, has been extensively trialled in the US and Canada (Table 2.2). The transgenic traits expressed most commonly are herbicide tolerance, but include many others such as tolerance to shade, pests and diseases, drought, salt, heat and aluminium. Most field trial applications have been made by Monsanto, Scotts and Rutgers University.

The most commercially advanced GM grass material comprises herbicide tolerant *Agrostis*. Scotts and Monsanto have made a joint application to APHIS for the deregulation of their ASR368 line of glyphosate tolerant *A. stolonifera*. This application is currently pending. *A. stolonifera* is known to hybridise with eight species of *Agrostis* and three of *Polypogon* in the US. In addition, there are a further 20 *Agrostis* species in the US for which the hybridisation potential with *A. stolonifera* is unknown. It is a fast growing perennial grass that can spread vegetatively via stolons with wind-pollinated flowers and small, wind and water dispersed seed. It is used extensively in golf greens, municipal parks and lawns (APHIS 2004) where herbicide resistance would be a great advantage to reduce management requirements. There is growing public concern, however, over the potential deregulation of this grass due to its ability to hybridise. It is claimed that none of the potential recipient species for transgene escape via hybridisation with GM *A. stolonifera* are a serious weed problem, as they do not appear on the Federal noxious weed list (APHIS 2004). It is claimed that the transfer of herbicide resistance from *A. stolonifera* as a result of transgene escape has the potential to generate a new set of problem weeds in the US. However, the Weed Science Society of America does not consider this to be the case as glyphosate is not commonly used to control *Agrostis* (Banks *et al.* 2004).

2.3.6.2 *Festuca* and *Lolium*

There are three species of *Festuca* and *Lolium* for which GM field trial applications have been made (Table 2.2). These species are all important forage grasses in the UK and Europe (Smartt & Simmonds 1995). *Festuca arundinacea* is wind pollinated and highly self-incompatible. It is native in Europe and will hybridise with all four of the other broad-leaved fescue species present in Europe. In addition, *F. arundinacea* will also hybridise with *Lolium perenne* and *L. multiflorum*, both of which have had GM field trials (Table 2.2). These two *Lolium* species are also native and will inter-cross freely between themselves and with the other three species of *Lolium* that occur in Europe (Tutin *et al.* 1964-1980).

F. arundinacea has been trialled in France and the US for herbicide tolerance, drought tolerance and disease resistance. Trials were also conducted in France with

lines having altered lignin biosynthesis. GM *L. perenne* lines have been trialled in the Netherlands to test genes inhibiting flowering; in the US drought and salt tolerant varieties have been trialled. The Noble Foundation in the US has been trialling *L. perenne* and *L. multiflorum* lines with transgenically reduced pollen allergens.

2.3.6.3 Other grasses

The remaining grass species with GM field trial applications are Canary grass (*Phalaris canariensis*), Russian wildrye (*Psathyrostachys juncea*) and St. Augustine grass (*Stenotaphrum secundatum*) (Table 2.2).

There are several species of *Phalaris* native to Europe and although *Phalaris canariensis* is introduced, it is widely naturalised in the Mediterranean regions of Europe. There has been one field trial of GM *P. canariensis* in Canada for a cultivar developed with herbicide tolerance. It has been cultivated as a minor cereal in the past but is now mainly cultivated for birdseed.

Psathyrostachys juncea does not have any related species in Europe, but occurs in the eastern border states of Europe in Russia and Belorussia. It is native to the steppes and deserts of Russia and China and is very tolerant to cold, drought, and to fire. It is also moderately tolerant of flooding. Individual plants are very long lived and are planted in the US for soil improvement, stabilisation and rehabilitation. As a forage grass it is nutritious and digestible (Taylor 2005). GM field trials of *P. juncea* remain at the experimental stage with the Noble Foundation trialling lines containing marker genes.

Stenotaphrum secundatum is introduced to Europe and so there are no related species present. The species is naturalised in some habitats along the seashores of the Mediterranean (Tutin *et al.* 1964-1980; Stace 1975). It is widely used as a lawn grass in parts of the US and can be used for erosion control. Scotts have conducted field trials of GM *S. secundatum* in the US with glyphosate resistance and with altered growth rates and morphology (Table 2.2).

2.3.6.4 Summary of grasses

Of the eight species of grass that have GM varieties in development, six are native in Europe and pose a risk of transgene escape. *Agrostis stolonifera* is one of these native species and Monsanto and Scotts have a pending application for the deregulation of glyphosate tolerant *A. stolonifera* in the US. Of the remaining three species in field trials, two exist as naturalised, escape populations in some areas of the Mediterranean.

2.3.7 Ornamentals

Cultivation of ornamentals as cut flowers or as garden plants does not occur as a major activity in the agricultural statistics as it does not cover any significant area of land and is a specialised commercial activity. Gene escape from certain cultivated ornamentals to wild relatives is possible in Europe and has the potential to affect populations of numerous species that are endemic to regions of Europe.

2.3.7.1 Rose

Rosa is a large genus of over 100 species native to the north temperate regions of the world. There are 47 species of native roses across Europe, and innumerable introduced ornamentals (Tutin *et al.* 1964-1980). *Rosa* is a very complex genus with a unique self-incompatibility system in that many of the species have unbalanced gametes in terms of their chromosome complement. Hybrids are common between species and can produce morphologically different offspring from the parental phenotypes (Stace 2001).

One of the most innovative of recent GM ornamental products (Chandler & Lu 2005) is the development of a genetically modified 'blue rose' announced by a joint venture between Suntory and CSIRO (<http://www.csiro.au/index.asp?type=faq&id=bluerose&stylesheet=divisionFaq>) in April 2005. This product has been produced using a three-gene construct consisting of a pansy gene encoding flavonoid 3'5'-hydroxylase, an

RNAi construct to inhibit the endogenous dihydroflavinol reductase (DHR) and a novel DHR gene from iris (Figure 2.7).

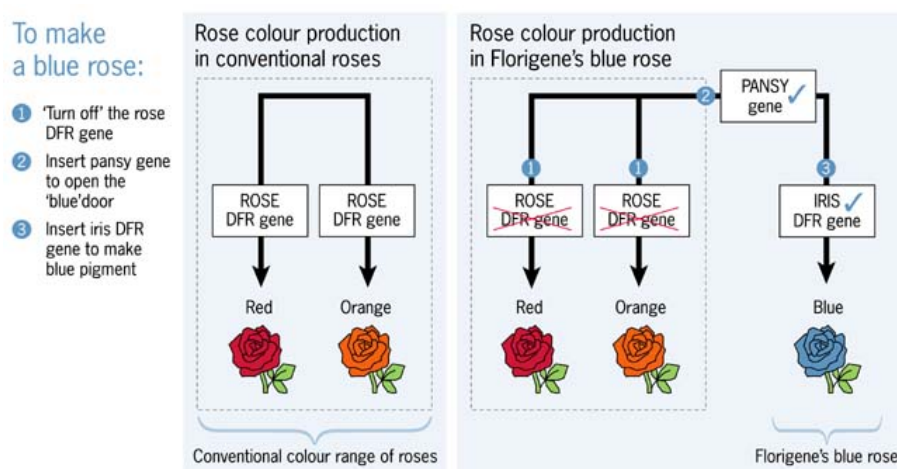


Figure 2.7 Method for production of blue rose

The plant expresses the blue pigment delphinidin, produced by introduction of the gene encoding the enzyme flavonoid 3'5'-hydroxylase from pansy and down regulation of the endogenous dihydroflavinol reductase gene. (Source: <http://www.florigene.com/news/news.php>)



Figure 2.8 Transgenic blue roses (Source: <http://www.florigene.com/news/news.php>)

This plant and its derivatives (Figure 2.8) may possibly become the first commercial product using RNAi technology. However, it is likely to be several years before this product goes through the regulatory process required prior to commercial release.

Hybrid roses have also been transformed to confer disease resistance (Marchant *et al.* 1998; Li *et al.* 2003b). Relatively few field trial applications have been made for GM roses and these have been in the US and Australia (Table 2.2).

2.3.7.2 Carnation

Forty one GM field trial applications have been made for carnations (*Dianthus* spp.) in Australia, Japan, Mexico and The Netherlands (Table 2.2). Florigene, now part of the Japanese Company Suntory, have been selling GM varieties of *Dianthus caryophyllus* with modified flower colour (Fukui *et al.* 2003), under the names 'Moondust', 'Moonshadow', 'Moonlite', 'Moonshade' 'Moonqua' and 'Moonvista' (Figure 2.9) since 1995 in Australia and 1997 in Europe. During this period, they have developed a considerable patent portfolio covering most of the anthocyanin/flavonoid pathway genes that control flower colour in petunia and numerous homologues from a diverse number of species, as well as methods for transformation of several ornamentals (Table 2.12).



Figure 2.9 Transgenic carnations with modified flower colours (Source: www.florigene.com)

The regulatory evaluation of this material has considered the occurrence of the many *Dianthus* species across Europe. This genus is native to the Mediterranean and there are 115 species present across Europe, of which there are 92 endemic taxa (either species or subspecies) (Tutin *et al.* 1964-1980). Most species are cross compatible but hybridisation is minimal, as the different species tend to be geographically isolated. Where species are sympatric, hybridisation is seen to occur (Stace 1975). This geographic isolation may prevent any transgene spread from population to population, however, the GM varieties of *D. caryophyllus* used as cut flowers are largely male sterile and there is no history of pollen dispersal or hybridisation with other *Dianthus* species despite many decades of growth throughout the world.

Table 2.12 Florigene patent portfolio

Roses	
US 5792927	Genetically transformed rose plants and methods for their production
US 5480789	
WO 9200371	Rose plants and methods for their production and genetic transformation
US 5530182	Methods for production of hybrid rose plantlets from rose somatic embryos
Carnations	
AU 703841	Transgenic carnations exhibit prolonged post-harvest life
WO 9635792	
WO 9217056	Carnation plants and methods for their transformation and propagation
US 5589613	
Chrysanthemums	
US 5567599	Method for producing transformed chrysanthemum plants
WO 9203041	
General Change in Pigmentation	
WO 9732023	Genetic sequences encoding flavonoid pathway enzymes for use in the genetic manipulation of flower pigments

2.3.7.3 Statice

Another ornamental species native to Europe is statice (*Limonium* spp.). There are 87 species of *Limonium* in Europe, of which 79 are endemic taxa (species or subspecies) (Tutin *et al.* 1964-1980). Although hybridisation between species is less common owing to the presence of a self-incompatibility mechanism, hybrids between species have been recorded in the UK.

Limonium species have been transformed to alter their form to induce dwarfing and early flowering, and in a more experimental study, to have green fluorescent petals by the insertion of the green fluorescent protein gene GFP (Mercuri *et al.* 2001a; b). Field trial applications have been made in Italy (Table 2.2).

2.3.7.4 Other ornamentals

Most other ornamental species for which GM field trial applications have been made [chrysanthemum, orchids, gladiolus, pelargonium, petunia, African violet, cape marigold, *Lisianthus* (Bradley *et al.* 2000)] represent less risk, as they are introduced species with no wild relatives present in the European flora. Some ornamental

species have escaped cultivation, however, and may become locally naturalised representing some potential for transgene spread.

2.3.7.5 Summary of ornamentals

Carnations are the most developed transgenic ornamental with GM varieties commercially available in some parts of the world. This species, together with rose (also under GM development), both have wild relatives present in the European flora. However, cultivated *Dianthus* species are unlikely to hybridise with wild species. Many other ornamental species with GM varieties under development are not native to Europe and present minimal opportunities for transgene escape.

2.3.8 Summary of European crops with potential for transgene escape

These crops are all cultivated in Europe, have GM varieties in development and could hybridise with wild/naturalised relatives in the European flora:

- **major crops** wheat, barley, oilseed rape, durum wheat, sunflower, oat, sugar beet, grapes
- **fodder crops** alfalfa/lucerne, barrel clover, lupin
- **forestry species** silver birch, sweet chestnut, Norway spruce, Scots pine, poplar, elm
- **orchard crops** olive, apple, plum, cherry, pear, walnut
- **grasses** *Agrostis canina*, *A. stolonifera*, *Festuca arundinacea*, *Lolium perenne*, *L. multiflorum*
- **ornamentals** rose, statice

3. Review of Containment Methods of Conventional Crop Species

The incentive for containment of transgenic crops, especially those producing pharmaceutical compounds (see Section 4), derives principally from the recognition of the difficulties of maintaining separation of such crops from their conventional equivalent. The most well known example of this problem is the so-called 'Starlink' episode in 2000 (<http://pewagbiotech.org/resources/issuebriefs/starlink/starlink.pdf>), where material from a non-approved line of *Bt* corn was found in processed food products in the US. This was followed in 2002 by identification of corn expressing trypsin as 'volunteers' in the following year's soybean crop. This latter incident led to the incineration of 63 ha of crops from around the site involved. In addition there are widespread concerns regarding the introgression of transgenes into native populations of wild species leading to undesirable ecological change, or into non-GM crops leading to the loss of non-GM or organic status of a crop.

There are a range of methods and technologies presently being investigated in an attempt to overcome this problem and to provide reassurance to the regulators and the public (Ellstrand 2003a). Amongst the approaches there has been extensive discussion of improved management oversight of the production process (Anon 2005, Wolt *et al.* 2004) but most activity is now focussed on more active methods to reduce or prevent the possibility of transgene dispersal (Gepts 2004).

These different alternative approaches, which range from physical isolation to complex molecular technology, will be discussed below.

3.1 Physical separation

The simple physical separation of the transgenic crop from its non-GM equivalent has been proposed as the most obvious method of maintaining purity and avoiding contamination. In its simplest form this includes the use of contained growth cabinets and greenhouses but much larger scale contained facilities have been developed. This was first exemplified by a project in which transgenic tobacco expressing a human recombinant glycoprotein B (gB) of human cytomegalovirus were grown in an underground copper and zinc mine in Flin Flon, Manitoba, Canada (Tackaberry *et al.* 2003). The company involved, Prairie Plants Systems Inc., (<http://www.prairieplant.com/home.htm>) have operated the facility, 360 metres below the ground, for 13 years during which time research has been conducted on over 500 plant species including various softwood and hardwood species, culinary herbs, nutraceuticals, ornamentals and agricultural crops (Figure 3.1). Examples include species of *Nicotiana*, *Taxus*, *Maize*, *Brassica*, *Populus*, *Echinacea*, and *Ocimum*. The company has also been awarded a five-year contract by Health Canada for the development of comprehensive operations for the cultivation and fabrication of marijuana in Canada. In 2000, operations were extended to the USA where the first underground chamber for the U.S. subsidiary, SubTerra LLC, was constructed and commenced operations in White Pine, Michigan. In the same year, SubTerra LLC was contracted for the growth, production and harvest of transgenic tobacco seed used in cancer research.



Figure 3.1 Underground growth facilities of Prairie Plant Systems, Inc
(Source: <http://www.prairieplant.com/home.htm>)

Since that time, interest in this approach has increased significantly. For example, the advantages of growing these crops underground are the theme of a submission from Numedloc Inc. to APHIS in 2003 (<http://www.fda.gov/ohrms/dockets/dailys/03/Jan03/011603/8004ac94.pdf>). In this response to Docket Number 02D-0324 (Guidance for Industry - Drugs, Biologics, and Medical Devices Derived from Bioengineered Plants for Use in Humans and Animals, Draft Guidance) the company point out the wide availability of underground chambers, principally limestone mines, across the US. It is stated:

‘The largest single mine we have examined has 1,600 acres of mineral depleted useable space. Several states have inventories of developable space. A single mine in a metropolitan area or serving a specific industry (steel, etc.) may create >40 acres of new space per year. Martin Marietta, the largest limestone producer in the US has operations in over 350 locations across the US. They and other companies can actually mine limestone deposits to spec’s to create large-scale underground production facilities. Limestone mines are typically at depths between 50 and 200 feet below the surface, have temperatures ranging between 50 and 70°F, and do not contain minerals such as Cu or Pb that represent direct health risks to workers.

‘Underground limestone mines offer significant advantages in environmental control for bioengineered crop production relative to the aboveground environment and regarding exposure of both workers and the public to undefined risks. Unlike greenhouses (including double envelope designs) and aboveground windowless facilities, underground mines are unaffected by wind, hail, snow or ice storms, tornados, fire, etc. Mineral-depleted limestone mines offer a secure protected biosecurity environment which lends itself to production spaces engineered on the clean corridor/dirty corridor concept widely used in animal research facilities, industrial clean rooms, etc. An underground facility can use the mass of the rock as a thermal sink for heat produced by electric lighting and avoids environmental heat gain from sunlight and the environment.’

This approach has since been developed by Controlled Pharming Ventures (<http://www.controlledpharming.com/>) a company founded by Doug Ausenbaugh of Purdue University. Their production facility is located in a 50 acre depleted limestone quarry in Marengo, Crawford County, Indiana. In a transcript of a UDSA meeting with various stakeholders held in February 2004 (<http://www.aphis.usda.gov/>

brs/stakeholder/Controlled_Pharming_Ventures.pdf) it is stated by Ausenbaugh that for IP reasons their first crop would be corn, followed by alfalfa, tomatoes, and leafy vegetables. In a related press article (<http://www.impactlab.com/modules.php?name=News&file=article&sid=5767>) John Turner, director of policy coordination in the USDA's Biotechnology Regulatory Services branch, is quoted as saying 'We probably wouldn't have regulatory authority inside a contained facility such as a mine'. Similarly, as part of their own web site advertising it is stated:

'The contained nature of our production system removes the USDA/BRS field testing permit requirement, which can be cumbersome', and

'The contained nature of our production system provides an exemption to the USDA's permitting requirements for field tests, dramatically reducing the time to market.'

3.1.1 Summary of physical separation

The physical containment of crops includes growth chambers and greenhouses and large-scale underground facilities. The contained growth of transgenic plants under such conditions is commercially attractive as it requires considerably less regulation, as there is no intended release into the environment.

3.2 Biological Containment

Several publications include some discussion of the range of methods under development to reduce or eliminate the transfer of transgenes from genetically modified plants. Thorough summaries are provided in several publications (Lu 2003; Gleba *et al.* 2004; Davison 2005; Gressel & Al-Ahmad 2005b), and the relevance of these approaches in the context of invasive ornamentals is discussed by Li *et al.* (2004).

The term molecular containment includes natural mechanisms that are the result of the biology of the plant, and any mechanism that is imposed by a recombinant DNA technology.

3.2.1 Natural Genetic Containment

The biology of certain plant species provides opportunities through careful management to eliminate the potential for pollen development. This would apply mostly to molecular pharming activities that use of the vegetative parts of the plant to produce pharmaceutical products and there is no need for pollination and seed development. For example, materials produced in the vegetative parts of alfalfa or tobacco can be harvested before flowering. (See section 4 of this report for a review of molecular pharming technologies). An alternative to the standard genetic engineering method of inserting genes into chromosomes is to insert genes into chloroplasts.

3.2.2 Plastid Transformation

3.2.2.1 Plastid biology

It is claimed that transgene flow from plants to wild relatives can be restricted if the transgene is targeted to the chloroplast DNA rather than to the nucleus, since in some angiosperm species plastid genes are almost entirely maternally inherited (Daniell *et al.* 1998; Heifetz 2000; Bock & Khan 2004; Daniell 2005). Prior to

pollination and fertilisation, egg cells develop and mature inside the gynoecium and each of these cells contains viable plastids. During pollen development in the anther, plastid DNA is usually lost from sperm cells when the pollen cells mature. Following pollination, sperm cells are delivered to the ovary and the embryo is formed from the fusion of a sperm cell and an egg cell. During this process, the nuclear genomes of the sperm and egg cell fuse and the plastids of the egg cell remain inside the zygote cell to become the plastids of developing embryo and of the next generation.

Plastid transformation technology is an attractive methodology for the genetic engineering of plants, as a typical plant cell contains around 100 chloroplasts, and each chloroplast contains around 100 identical genomes. This means that a single gene may be represented 10,000 times within one cell (Bendich 1987). Genes encoded in repeat regions of the chloroplast genome of higher plants may be encoded 20,000 times, thus resulting in high levels of transgene expression and recombinant products (Maliga 2004; Grevich & Daniell 2005).

Some workers have criticised the argument that plastid transformation prevents gene flow via pollen. Although transmission of plastid DNA via pollen is rare, it is not absent in all plants and some viable plastids may pass from the pollen tube into the zygote along with the sperm cell during fertilisation (Cummins 1998; Lu 2003; Wang *et al.* 2004a). This process of paternal or bi-parental inheritance of plastids is known from species such as rye (Mogensen & Rusche 2000) and can be examined in other species using PCR techniques (James *et al.* 2001).

There is also the possibility of gene flow from the chloroplast to the nucleus, a process that may represent an additional pathway for transgene escape. It is argued that any genetic construct designed for chloroplast expression would not normally function if transferred to the nucleus. In order for transferred genes to become functional in the nuclear genome, they must undergo complex changes requiring several million years of evolution (Grevich & Daniell 2005).

Transgene containment via plastid transformation only prevents gene flow via pollen; it does not prevent the formation of hybrids from the pollen of wild species fertilising the transgenic crop plant or prevent escape via seed from the crop itself during

harvest and transport. The persistence of feral crop plants expressing the transgene provides additional opportunities for hybrid formation with wild populations. If such hybrids persist to flowering, any seed of this plant will also contain the functioning transgene regardless of the pollen parent resulting in progeny that are transgenic and potentially weedy. The suitability of plastid transformation for transgene containment requires regulatory assessment on a case by case basis.

3.2.2.2 Plastid transformation methodology

Selection for chloroplast transformation generally uses antibiotic resistance genes (spectinomycin) as selectable markers (Golds *et al.* 2004). The use of antibiotic resistance is a concern to the public acceptance of GM and to the regulation of crops (Gay & Gillespie 2005; Goldstein *et al.* 2005), as the escape of antibiotic resistance to related species and micro-organisms cannot be excluded. Technologies have been developed to remove the marker gene by homologous recombination (Klaus *et al.* 2004; Maliga 2005). However, a new selection method has been devised to obviate the use of antibiotic selection in chloroplast transformation. This uses the betaine aldehyde dehydrogenase gene of spinach, which converts the toxic betaine aldehyde into non-toxic betaine glycine, an osmoprotectant (Daniell *et al.* 2001b). This method is claimed to provide a more efficient means of selection and could theoretically be used to enhance the stress tolerance of the plant (Grevich & Daniell 2005).

Chloroplast engineering continues to develop and an inducible plastid promotion system has been developed recently. Mühlbauer and Koop (2005) have demonstrated an inducible plastid promoter in tobacco expressing the green fluorescent protein marker gene using an isopropyl- β -D-thiogalactopyranoside (IPTG) chemical regulator. The system is even effective on post-harvest leaves, offering further containment potential for recombinant protein and bio-pharming production. As the inducer reagent can be applied post-harvest it is not released into the field, thus the ecological implications of chemical spray applications are removed. In addition, less inducer reagent is required as leaves may be submerged, thereby reducing costs and ensuring sufficient inducer application across the entire sample. This enables transgenic plants to be grown in the field with the complete

suppression of the transgene product during the whole growth phase of the plant with induction taking place in an enclosed and controlled environment (Mühlbauer & Koop 2005). Such a system offers a great potential for bio-pharming of potentially hazardous or valuable products. A related system using an ethanol-induced promoter has also been applied to the production of polyhydroxybutyrate in transplastomic tobacco (Lossl *et al.* 2005).

Transgene expression in the plastid is not suitable for all applications as the proteins expressed by the chloroplast remain in the chloroplast. This is well suited to the accumulation of biosynthetic products that may cause a negative effect to the plant if they were in the cytoplasm and makes plastid expression a useful tool for bio-pharming (Daniell *et al.* 2005) (Table 3.1). Transgenes aimed at modification of the physiology of the plant are not suitable for plastid expression as the products of the plastid are not glycosylated (Gleba *et al.* 2004). In addition, reliable chloroplast transformation can currently only be performed on tobacco as there is a lack of complete chloroplast genome sequences in most crop species (Gleba *et al.* 2004; Grevich & Daniell 2005), though see below for information on other species.

Table 3.1 Expression of vaccine antigens and biopharmaceutical proteins in the plastid
(Adapted from Grevich and Daniell 2005)

Therapeutic proteins	Gene	Total soluble protein %	Reference
Elastin derived polymer	<i>EG121</i>	None detected	Guda <i>et al.</i> 2000
Human somatotropin	<i>hST</i>	1-7	Staub <i>et al.</i> 2000
Cholera toxin	<i>CtxB</i>	4	Daniell <i>et al.</i> 2001a
Antimicrobial peptide	<i>MSI-99</i>	Not tested	DeGray <i>et al.</i> 2001
Insulin-like growth factor	<i>IGF-1</i>	33	Daniell <i>et al.</i> 2004a
Interferon alpha 5	<i>INFa5</i>	Not tested	Torres 2002
Interferon alpha 2b	<i>INFa2B</i>	19	Daniell <i>et al.</i> 2004b
Human serum albumin	<i>has</i>	0.02-11.1	Fernandez-San Millan <i>et al.</i> 2003
Interferon gamma	<i>IFN-g</i>	6	Leelavathi & Reddy 2003
Monoclonal antibodies	<i>Guy's 13</i>	Not tested	Daniell <i>et al.</i> 2004b
Anthrax protective antigen	<i>pag</i>	4-5	Chebolu & Daniell in press
Plague vaccine	<i>CaF1~LcrV</i>	4.6	Singleton 2003
CPV VP2	<i>CTB-2L21</i>	31.1	Molina <i>et al.</i> 2004
CPV VP2	<i>GFP-2L21</i>	22.6	Molina <i>et al.</i> 2004
Rotavirus VP6	<i>vp6</i>	0-3.0	Birch-Machin <i>et al.</i> 2004
Tetanus toxin	<i>Tet C</i>	10-25	Tregoning <i>et al.</i> 2003

A special feature of chloroplast engineering is that chloroplasts are present in large numbers in leaves, but not in roots. Thus, its application has some limitations and it

is not certain how effective the insertion of transgenes into chloroplast DNA would be for phytoremediation purposes (Davison 2005).

3.2.2.3 Commercial applications

Tobacco has been genetically modified using plastid transformation technology to confer insect resistance, herbicide tolerance, disease resistance and heavy metal tolerance in addition to the expression of a wide range of vaccine antigens and pharmaceutical proteins (see section 4). Recent permits for field trials of chloroplast transformed tobacco plants in the United States have been issued by APHIS (Animal and Plant Health Inspection Service, USDA) for the following material:

Phenylalanine ammonia lyase: University of Kentucky (in collaboration with Icon Genetics, Munich) issued 7 June 2005

Gene construct not detailed: Chlorogen, Inc. issued 19 May 2005

Human serum albumin: Chlorogen, Inc. issued 25 May 2004 and 15 May 2003

Insulin like growth factor: Chlorogen, Inc. issued 25 May 2004

Interferon: Chlorogen, Inc. issued 25 May 2004

Mullerian inhibiting substance: Chlorogen, Inc. issued 25 May 2004

In addition, an application was made to APHIS in 2004 by Chlorogen, Inc to field trial tobacco expressing cholera toxin B, and protective antigens from *Bacillus anthracis* and *Yersinia pestis* and later withdrawn.

Chlorogen, Inc (<http://www.chlorogen.com/>) is a commercial concern based upon the chloroplast transformation technology developed by Henry Daniell (Daniell *et al.* 2005) and is one of the foremost companies specializing in this technology (Maliga 2004). The company has a considerable intellectual property portfolio (Table 3.2). In November 2004, Chlorogen signed a joint development and supply agreement with Sigma-Aldrich Fine Chemicals, which is expected to produce the first commercial products from chloroplast transformation technology. Sigma-Aldrich will fund an undisclosed portion of Chlorogen's efforts to produce four specific proteins in tobacco plants. The proteins will be sold to the reagent and cell culture markets and have pre-identified applications as active pharmaceutical ingredients.

Table 3.2 Chlorogen, Inc patent portfolio

Inventors	Date	Title	Patent
H. Daniell, B.A. McFadden	2004	Chloroplast genetic engineering	US 6680426
H. Daniell, B.A. McFadden	2003	Chloroplast genetic engineering	US 6642053
H. Daniell, K. Wycoff	2001	Production of antibodies in transgenic plastids	WO 0164929
H. Daniell	2001	Multiple gene expression for engineering novel pathways and hyper-expression of foreign proteins in plants	WO 0164024
H. Daniell	2001	Pharmaceutical proteins, human therapeutics, human serum albumin, insulin, native cholera toxin B subunit in transgenic plastids	WO 0172959

It was reported recently, that in a move towards developing this production system in tobacco, Chlorogen has had discussions with Missouri tobacco growers and with Kansas City area biotech leaders. The company requires financial backing to build a processing plant for products expressed in tobacco. At present, only Southeast Missouri State University and its host city, Cape Girardeau, have made offers to help with processing and research buildings and negotiations are under way for a Chlorogen plant there.

Other companies developing chloroplast production platforms include Icon Genetics (<http://www.icongenetics.com/html/02.htm>) who have a proprietary version of the plastid transformation technology (Figures 3.2, 3.3) and have a large patent portfolio (Table 3.3). [Note added in proof: Icon Genetics was acquired by Bayer Innovations GmbH, a subsidiary of Bayer AG, on 9 January 2006].

3.2.2.4 European perspective

Tobacco is cultivated in the southern states of Europe and ranks 50 in terms of area (Table 2.6). It is also cultivated in the overseas departments of Reunion and the Canary Islands (Table 2.8). As tobacco is native to South and Central America, there are no native wild relatives in the European flora. There are, however, cultivated species that have escaped from cultivation and become locally naturalised in some countries; these include *Nicotiana tabacum* (cultivated tobacco), *N. rustica*, (formerly cultivated for tobacco), *N. glauca* and *N. alata* (both introduced as ornamentals). The presence of naturalised *N. tabacum* represents a potential escape route for transgenes into the environment.

Table 3.3 Icon Genetics patent portfolio.

Patent number	Date	Title
WO 05054478	16 June 2005	Controlling Gene Expression in Plastids
WO 05049839	2 June 2005	RNA-Virus-Derived Plant Expression System
WO 04108934	16 Dec 2004	Safe Production of a Product of Interest in Hybrid Seed
WO 04101797	25 Nov 2004	Process of Producing a Plastid-Targeted Protein in Plant Cells
WO 04067748	12 Aug 2004	Plant Transformation with in vivo Assembly of a Trait
WO 04067749	12 Aug 2004	Plant Transformation with in vivo Assembly of a Sequence of Interest
WO 04046359	3 June 2004	Method of Controlling Processes in Plants or Plant Cells
WO 04046360	3 June 2004	Method of Controlling Cellular Processes in Plants
WO 04046361	3 June 2004	Method of Controlling a Cellular Process in a Multi-Cellular Organism
WO 04015115	19 Feb 2004	Plastid Transformation Using Modular Vectors
WO 03102197	21 March 2003	Transgenic Plants with Controlled Distribution of a Trait to Progeny
WO 03020927	13 March 2003	Creation of Artificial Internal Ribosome Entry Site (IRES) Elements
WO 03020928	13 March 2003	Identification of Eukaryotic Internal Ribosome Entry Site (IRES) Elements
WO 03020938	13 March 2003	Method of Protein Production in Plants
WO 03012035	13 Feb 2003	Commercial Use of Arabidopsis for Production of Human and Animal Therapeutic and Diagnostic Proteins
WO 03004658	16 Jan 2003	Gene Expression in Plastids Based on Replicating Vectors
WO 03001900	9 Jan 2003	Process of Controlled Shuffling of Chromosome Fragments for Plant Breeding
WO 02101006	19 Dec 2002	Production of Proteins in Plants
WO 02101060	19 Dec 2002	Processes and Vectors for Producing Transgenic Plants
WO 02096192	5 Dec 2002	Process of Producing Environmentally Safe Transgenic Organisms
WO 02097080	5 Dec 2002	Amplification Vectors Based on Trans-Splicing
WO 02088369	7 Nov 2002	Processes and Vectors for Amplification or Expression of Nucleic Acid Sequences of Interest in Plants
WO 02083867	24 Oct 2002	IRES Enabled Gene Trapping in Plants
WO 02079481	10 Oct 2002	Method of Encoding Information in Nucleic Acids of a Genetically Engineered Organism
WO 02077246	3 Oct 2002	Site-targeted Transformation Using Amplification Vectors
WO 02068927	6 Sept 2002	Using Viruses to Detect or Purify Proteins
WO 02068664	6 Sept 2002	Recombinant Viral Switches for the Control of Gene Expression in Plants
WO 02057466	25 July 2002	Processes and Vectors for Plastid Transformation of Higher Plants
WO 02055651	18 July 2002	Processes and Vectors for Plastid Transformation
WO 0246440	13 June 2002	Processes and Vector Systems for Producing Transgenic Plants
WO 0229068	11 April 2002	Vector Systems for Plants
WO 0170939	27 Sept 2001	Methods for Transforming Plant Plastids and Making Transplastomic Plants
WO 0111020	15 Feb 2001	Method of Making Plant Artificial Chromosomes
WO 0070019	23 Nov 2000	Process of Rapid Variety-Independent Plant Transformation
WO 9855636	10 Dec 1998	Recombinant Construct for Enhancement of Gene Expression in Plants
WO 9854342	3 Dec 1998	Methods for Coexpression of more than one Gene Using at least one Internal Ribosome Entry Site (IRES)

3.2.2.5 Other species

Although tobacco remains the species most amenable to chloroplast transformation, recent successes have been achieved with *Petunia* (Zubko *et al.* 2004; US Patent Application 040199937), cotton (Kumar *et al.* 2004b), carrot (Kumar *et al.* 2004a) (Figure 3.5), tomato (Ruf *et al.* 2001; US Patent Application 040237131), soybean

(Dufourmantel 2004), lettuce (Lelivelt 2005), *Brassica* (Nugent *et al.* 2006; US Patent 6891086) and another crucifer species, *Lesquerella fendleri* (Skarjinskaia *et al.* 2003; US Patent Application 030200568), but stable integration of transgenes into the chloroplast of rice (Khan and Maliga 1999) and oilseed rape (Hou *et al.* 2003) have so far been unsuccessful.

Food crops with developed plastid transformations include tomato, carrot and soybean and these are all important crops in European agriculture (Table 2.6). Carrots are native to the Mediterranean and cultivated carrots will hybridise with their wild relatives, of which some in Europe are endemic (Tutin *et al.* 1964-1980). Carrots are biennial, however, and under normal circumstances should not flower under cultivation as the root develops during the first year's growth and the inflorescence in the second year. In addition, carrots are thought to undergo strict maternal inheritance of the chloroplast (Vivek *et al.* 1999). Transformation of carrots via the plastid genome has been used to confer high levels of salt tolerance but this material has not yet reached field trials.

Tomato and soybean represent a low-risk for transgene escape to wild relatives in Europe as there are no related species in the European flora (Table 2.10), although crop-to-crop gene flow remains a possible escape for transgenes for all crop species. The development of plastid transformed tomato and soybean cultivars remain in the experimental stage as successful plastid transformation of these species has only been developed recently (Grevich & Daniell 2005; Koya & Daniell 2005). Similarly for cotton, chloroplast transformation technologies have been developed as alternative to nuclear transformation as a means of reducing the public concern against GM cotton (Grevich & Daniell 2005). Cotton is an important crop in Europe ranking 28 (Table 2.6), but it also represents a low risk of transgene escape to wild relatives in the European flora (Table 2.10).

3.2.3 Genetically Engineered Gene Containment

3.2.3.1 Conditional Lethality

Transgenic plants may be engineered to contain a conditionally lethal gene that is capable of converting a biologically inert toxin precursor into a toxic compound. As the gene is only expressed in transgenic plants, spraying fields with the precursor only kills the transgenic plants and non-transgenic plants are unaffected. Such chemical compounds based on the herbicides glyphosate and glufosinate have been developed by Monsanto and Hoechst respectively.

Kriete *et al.* (1996) isolated a gene from *E. coli* that converts non-toxic *N*-acetyl-L-phosphinothricin (*N*-ac-Pt) into cytotoxic L-phosphinothricin (Pt, glufosinate). Tobacco plants transformed with the gene *argE* died following application of *N*-ac-Pt whereas untransformed plants showed no reaction. This gene has also been employed to induce male sterility by a process in which the *argE* gene is fused to a DNA fragment known to function exclusively in the tapetum of the pollen grain. The release of glufosinate in the developing anthers following *N*-ac-Pt application resulted in male-sterile plants. In the absence of *N*-ac-Pt application plants showed normal pollen development and full fertility (Kriete *et al.* 1996).

Other genes have been isolated that can be used to the same effect. For example, the *pehA* gene also works on the basis of known herbicide chemistry and converts the non-toxic phosphonate of glyphosate into glyphosate (US Patent 5254801) leading to induced tissue damage and death of the plants containing the transgene converter. Similarly, the *iaaH* gene converts non-toxic levels of naphthalene acetamide into toxic levels of the auxin naphthalene acetic acid (Klee *et al.* 1987). Other such systems have been developed for negative plant selection and inducible male sterility (Kobayashi *et al.* 1995; O'Keefe *et al.* 1994; Perera *et al.* 1993; patents WO 9204454, WO 03003816).

Examination of the EU, USDA (APHIS) and OECD databases of GM field trials (<http://biotech.jrc.it/deliberate/gmo.asp>; <http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>;

<http://webdomino1.oecd.org/ehs/biotrack.nsf>) does not identify any applications for deliberate release of plants transformed with genes used for conditional lethality. Complete information of transgene constructs is not available for all field trial applications, however, owing to business confidentiality.

3.2.3.2 Inducible Promoters

Transgenes can be activated by an artificial stimulus such as a chemical spray. In the absence of the stimulus, the transgene would not express the trait and there will be no conferred advantage to wild or weedy relatives receiving the transgene through hybridisation.

Many different types of promoter elements have been described and those that result in high levels of expression or limit expression to certain organs such as leaves or seeds are valuable for molecular farming purposes. Promoter elements can also be inducible and thus associated genes are only activated under certain conditions. Induction may be dependent on natural or physiological parameters such as heat, cold, day length, stage of development or may be dependent on the presence of a certain chemical or biochemical. Promoters that are specifically induced by applied chemicals can be used to drive expression of genes only when these chemicals are applied to the plants, thereby restricting the expression of the transgene to limited periods. For example, a crop may be sprayed to induce herbicide resistance prior to herbicide application. Several gene regulation systems have been developed that may be used to confer inducible herbicide resistance in plants. These include those induced or repressed by the antibiotic tetracycline, the 'Triple-Op' promoter (Gatz and Quail 1988) and the 'Top10' promoter (Weinmann *et al.* 1994); the GAL4-UAS promoter induced by the steroid glucocorticoid (Aoyama and Chua 1997) and the 'GRH' or similar promoter induced by the insect prohormone ecdysone (Martinez *et al.* 1999; Unger *et al.* 2002). There are also systems based on induction via copper (Mett *et al.* 1993; Granger & Cyr 2001), acetaldehyde (Junker *et al.* 2003) and the steroid dexamethazone (Tang *et al.* 2004). The most advanced of these technologies is the ethanol-induced '*alcA*' promoter (Caddick *et al.* 1998, Sweetman *et al.* 2002) developed by Syngenta to induce resistance to glyphosate (Patent WO 9706269). No records of field trials of crops

transformed with the *alcA* promoter can be found in the USDA, EU or OECD GM field trials databases.

Inducible promoters can also be used to activate expression of a site-specific recombinase enzyme that may excise the transgene prior to flowering (Daniell 2002). Such a mechanism would prevent passage of the transgene into the DNA of the developing gametes, thereby inhibiting movement from the crop to wild or weedy relative, or to non-GM crops. See section 3.2.3.11 for details of transgene excision.

Such technology is beneficial to traits that required on a transitory basis, but is limited for traits that require extended or constant expression such as insect or pathogen resistance, or production of a bio-pharmaceutical (Daniell 2002). This system is also likely to incur additional costs to the producer in terms of additional spray applications and chemical inputs. The nature of the chemical inducer would also require assessment and regulation, though there have several tests of systems involving the use of 'safeners' (DeVeylder *et al.* 1997). These are chemicals such as *N,N*-diallyl-2,2-dichloroacetimide (Patent WO 9301294), are used as additives in herbicide formulations (Davies & Casely 1999; Hatzios & Burgos 2004) and are thus already approved for use in the field. Some of these systems involve the use of the glutathione-S-transferase (GST) promoter (DeRidder *et al.* 2002). Stability of any such system would also need to be rigorously tested to ensure that there is no 'leakiness' of the promoters in the 'off' state, and sufficient levels of expression in the 'on' state following inducer application (Gleba *et al.* 2004).

In a similar way, promoters that are induced by certain physiological conditions such as injury and chopping after harvest can also be used to limit production of the farmed commodity until after the plants are actually harvested (Patent Application 030226163). This technology, known as MeGa-PharMTM was the focus of the company CropTech (no longer in business).

Containment technology based on inducible promoters appears to remain at the experimental stage with there being no confirmed evidence of crops that have been through field trials. Complete information of transgene constructs is not available for all field trial applications, however, due to business confidentiality.

The use of inducible promoters does not prevent transgene escape, but the unwanted side effects of escape of the trait are removed.

3.2.3.3 Engineered Male Sterility

Plant breeders have had a long interest in techniques to control hybridisation in plants. Breeding programs aim to stabilise and exploit beneficial traits or to limit and remove any unwanted characteristics. It is likely that prior to human intervention, most domesticated plants were capable of cross-pollination and possessed some kind of genetic self-incompatibility mechanism to promote out-crossing (Gleba *et al.* 2004). The desire for uniform, high-yielding crop plants has seen the selective elimination of self-incompatibility from many crop lines but the incompatibility genes and mechanisms remain in the wild populations, offering a naturally occurring mechanism for controlling cross-pollination in some crops (Gleba *et al.* 2004). In addition, systems developed by plant breeders to produce hybrid seed have included methods of inducing male sterility such as nuclear and cytoplasmic male sterility. Due to existing knowledge of these genes and mechanisms, there are a large number of methods to engineer male sterility in plants by recombinant means. Most transgenic methods involve the disruption or alteration of the pollen development process via programmed death (genetic ablation). Such methods often exploit tissue or cell-specific promoter elements that regulate genes encoding products that have disruptive or cytotoxic effects on developing pollen cells or structures required for normal pollen development. The disruptive agent may be a toxic protein (Kurek *et al.* 2002) or peptide (Guerineau *et al.* 2003; Lee *et al.* 2003), anti-sense construct (Zhang *et al.* 2001; Luo *et al.* 2000; 2004; Yan *et al.* 2000; Millar *et al.* 1999; Vandermeer *et al.* 1992), an enzyme (Ruiz & Daniell 2005; Tsuchiya *et al.* 1995), a nuclease (Moritoh *et al.* 2005), a ribonuclease (Bernd-Souza *et al.* 2000), or a plant growth regulator (Al-Ahmad & Gressel 2005; Huang *et al.* 2003a).

Probably the most commonly used system to disrupt pollen development and induce male sterility is the insertion of the gene encoding the highly destructive ribonuclease barnase. The gene is expressed under control of a tapetum-specific promoter, and thus acts only on cells in the anther during pollen development. Barnase-based male

sterility has been developed in tobacco (Zhan *et al.* 1996; Custers *et al.* 1997), rice (Zhan *et al.* 1996), maize (Liu *et al.* 2000), alfalfa (Rosellini *et al.* 2001), oilseed rape (Denis *et al.* 1993; Jenkins *et al.* 1999), tomato (Burgess *et al.* 2002), wheat (DeBlock *et al.* 1997), citrus (Li *et al.* 2002; 2003a) and birch (Lemmetyinen *et al.* 2001a; Lannenpaa *et al.* 2005a). This system has additional advantages in that a restorer system (barstar) has been developed that can counter the action of the barnase gene product. The barstar gene can be engineered into the crop line to restore male fertility if required. Barnase/barstar systems have been developed in *Brassica juncea* (Jagannath *et al.* 2002; Bisht *et al.* 2004), tobacco (Beals & Goldberg 1997), chicory and cauliflower (Reynaerts *et al.* 1993).

There have been numerous field trials of crops with male sterility engineered via barnase expression. In the US, there have been 121 field trials of crops expressing the barnase transgene to induce male sterility (<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>). The vast majority of these have been with maize (82), but there have also been numerous trials with oilseed rape (13) and *Brassica oleracea* crops (8). The other crops trialled are creeping bentgrass (*Agrostis stolonifera*), *Petunia*, lettuce, poplar, potato, chicory, tobacco and cotton. Up to 1999, there were 19 field trials in Europe of crops transformed with barnase to induce male sterility, the majority taking place in the Netherlands, but also in Germany, Belgium and the Czech Republic. The crops included sunflower, maize, chicory, potato, oilseed rape and wheat.

Several crops with male sterility conferred via barnase expression are approved for commercial release. In the US, APHIS approved release of two cultivars of oilseed rape (Aventis, AgrEvo), two cultivars of maize (AgrEvo, Plant Genetic Systems) and one of chicory (Bejo) and around 10% of GM oilseed rape grown in Canada has barnase engineered male sterility (Daniell 2002). In addition, oilseed rape line Ms8xRf3 of Bayer BioSciences expressing male sterility via barnase has a pending approval for the importation of seed for processing in the EU under notification C/BE/96/01, pending under directive 2001/18.

Alternative systems for inducing male sterility are frequently being identified. One recently reported system is based on expression of β -ketothiolase (Ruiz and Daniell

2005). This enzyme alters the course of fatty acids synthesis and accumulates in leaves and anthers. Tobacco lines transformed with the *phaA* gene that encodes β -ketothiolase were normal except for the male-sterile phenotype lacking pollen. Expression of β -ketothiolase disrupts the correct lipid metabolism required for pollen wall development and results in the collapse of the developing pollen grains. Restoration of normal fatty acid synthesis can be induced under continuous illumination; thus fertility can be restored resulting in viable pollen and large amounts of seed. This study represents the first engineered cytoplasmic male-sterility system in plants, offers a new tool for transgene containment for both nuclear and organelle genomes, and provides an expedient mechanism for F₁ hybrid seed production. The results are discussed by Khan (2005).

Similarly, Al-Ahmad and Gressel (2005) have recently investigated the utility of the gibberellic acid insensitive Δgai gene from *Arabidopsis* in inducing male sterility in tobacco. This sterility system is also reversible by the application of kinetin (Al-Ahmad & Gressel 2005). In addition, Chrimes *et al.* (2005) have identified male sterility in wheat following transformation with the mitosis-regulating gene *Spcdc25*.

With the constant development of new methods to induce male sterility in plants, the prevention of gene flow from GM crops via pollen would appear to be a realistic and viable containment strategy in the near future. Containment via pollen is unidirectional, however, and does not prevent transgene escape via seed from the GM plant. If male sterile plants are pollinated by compatible weeds, their progeny could establish as weedy crop-wild populations carrying the transgenic trait of the crop. In addition, many types of naturally occurring male sterility are leaky, and sufficient testing is required to determine the stability of any engineered trait. The packaging of several transgenic traits such as herbicide tolerance and male sterility requires precise insertion into the genome to reduce the risk of separation in meiosis due to crossing over and recombination of chromosomes. Consideration should also be given to planting strategies, as large scale planting of male sterile plants for seed production will require additional planting of pollen donor plants.

The expression of the toxic agent can be the result of a single gene or the result of two genes where the combined activity is required to achieve the disruptive effect.

Two-gene systems provide opportunities to devise sophisticated pollen control methods wherein bio-pharming plants would be able to self and cross with other bio-farming plants in the field, but would be unable to outcross due to the segregation of genes resulting in a lethal effect. The US company Ceres have developed one version of this technology (Mascia & Flavell, 2004; Patent Applications WO 04027038, US 050257293). This system provides a high level of expression of desired products coupled with a genetic containment strategy, and can be applied to plants expressing recombinant proteins in the seed or vegetative parts.

The Ceres' system is based on two components: the first is a yeast transcription factor under the control of a selected plant promoter; the second is an upstream activation sequence (UAS) controlling the expression of the foreign protein. Each component is engineered separately into a male sterile target line and a male fertile activation line. The male fertile line contains the yeast transcription factor in its pollen to activate production of the desired trait in the seed of the male sterile target line via the corresponding upstream activation sequence. The desired protein is then produced in the seed of the male sterile target line (Mascia & Flavell 2004).

Such a system is effective in species that readily cross-pollinate on a large scale. As the seed are harvested and processed to obtain the recombinant protein, seed germination and normal plant development are not required. As a result, the product may be accumulated in the seed at high levels. Indeed, a transcription factor that leads to abnormal embryo development is inserted into the male sterile target line to ensure that the seed produced will not germinate, thus preventing transgene escape via seed. The plants that contain the transgene expressing the novel protein produce no pollen, and the yeast transcription factor genes inserted into the male fertile activation line are commonly found in the food chain.

The genes used in the Ceres system are artificially inserted into the genome of the plant species used, and it is possible that non-native DNA will be transferred to wild relatives or non-GM crops. As such, these plants will still require regulation as genetically modified organisms. In addition, farmers will be required to plant out both types of plant in the field, ensuring a sufficient ratio of male fertile to male sterile target plants to ensure maximum seed return and yield.

Despite a varied and extensive number of methods of transgenically engineering male sterility in GM plants there are few systems other than barnase that have been extensively field trialled. Examination of field trial permits for the US of crops with male sterility engineered using a system other than barnase shows that there have been 31 applications. Details of the male sterility systems used are often not disclosed on these applications due to business confidentiality.

3.2.3.4 Genetic Use Restriction Technologies (GURTs)

Various systems of Genetic Use Restriction Technology (GURT) (for summary see:- <http://www.seedquest.com/forum/c/CollinsHarry/0.htm>; <http://cls.casa.colostate.edu/TransgenicCrops/terminator.html>) can be engineered into transgenic crops plants as a containment strategy and there are a substantial number of patent applications that describe various GURT concepts and elements (Appendix III). Two main types of GURT mechanisms have been developed to date: V-GURTs, that produce sterile seeds; and T-GURTs that only express their introduced trait if treated with a specific chemical inducer. The biological (Visser *et al.* 2001), economic (Eaton & van Tongeren 2002; Eaton *et al.* 2002) and social (Garí 2002; Lence & Hayes 2005) aspects of this subject have been reviewed in detail elsewhere and only a summary will be provided here.

The first type (V-GURT) restrict the use of the entire variety through interference with the reproduction process, and within this type, three subtypes of V-GURT can be distinguished (Visser *et al.* 2001):-

- 'In the first, the seeds are fertile, but through the application of a chemical a dormant 'lethal' gene can be activated. This gene inhibits full seed development. As a consequence, seeds are fit for consumption but infertile.
- In a second, a lethal gene is expressed in the seed, resulting in sterility. Breeders can apply a chemical compound that activates another gene to safeguard the fertility of the seed and the reproduction of the variety, but they will stop doing so before selling the seed.

- The third, and complementary, strategy concerns vegetatively reproducing crops, e.g. root and tuber crops and ornamentals. The described method involves prevention of growth during storage, which may be in the interest of breeders, growers and/or consumers. Normally, a gene that blocks growth is expressed, but growth can be restored by activation of a second gene. Regulation of hormone metabolism and function lies at the basis of this strategy.'

In contrast, in the T-GURT concept, one or more genes conferring a single trait are switched on or off at will through chemical inducers. The seed itself remains viable.

It is important to realize that, although current patent applications apply to plants, GURTs can be built into any organism, including farm animals and fish.

Sometimes known as 'Terminator Technology', seed sterility V-GURT systems have been much criticised in the media and elsewhere as they have been portrayed as a vehicle for large multi-national seed companies to suppress the freedom of farmers on the basis that it prevents them from saving and re-sowing seeds.

The original GURT patent (US Patent 5723765), entitled "Control of Plant Gene Expression", was granted in 1998 to the USDA and Delta & Pine Land, a cotton breeding company (<http://www.deltaandpine.com/>). The patent was based on research conducted under a Cooperative Research and Development Agreement (CRADA) signed in 1993 between Delta & Pine Land Company and the Agricultural Research Service, a subunit of the USDA. The European version of this patent (EP 775212) was granted on 5 October 2005. The seed lethality system proposed provides a method to protect against transfer of novel traits to other crops and plant species as it causes seed death at a late stage in seed germination (<http://www.biotech-info.net/howto.html>; <http://filebox.vt.edu/cals/cses/chagedor/terminator.html>).

There are three components to the specific seed-lethality mechanism described in this patent:

1. A bacterial tetracycline-responsive Tet repressor gene (from *Tn10*) under the control of a constitutive promoter. The repressor is inactivated in the presence of tetracycline.
2. A phage P1 site-specific recombinase gene (*cre*) under the control of a promoter that is subject to repression by the Tet repressor.
3. A gene coding for a cyto-toxic protein (a ribosomal inhibitor protein from *Saponaria officinalis*) expressed from a seed-specific plant promoter (late embryogenesis abundant, *LEA*), active only at a very late stage of seed maturation. The expression of this toxin gene is prevented by a DNA spacer sequence blocking its transcription. This latter sequence is bounded by the sites of action (*loxP*) of the site-specific recombinase *Cre*.

The construct is triggered by tetracycline, which acts as a specific stimulus. In the absence of the stimulus, the presence of the DNA spacer region in the gene coding for the cyto-toxic protein prevents its expression and allows seed germination to occur as normal. The application of the stimulus triggers the Tet repressor to activate the site-specific recombinase gene *cre*, which excises the DNA spacer region by acting on the *loxP* sites at either side. The result is a complete, uninterrupted gene encoding the cytotoxic protein, which is expressed at the specified late stage of seed maturation resulting in specific destruction of seed tissues.

A diagrammatic representation of such a method is shown below in Figures 3.2, 3.3 and 3.4 (adapted from [http://cls.casa.colostate.edu/TransgenicCrops/terminator diagram.pdf](http://cls.casa.colostate.edu/TransgenicCrops/terminator%20diagram.pdf)).

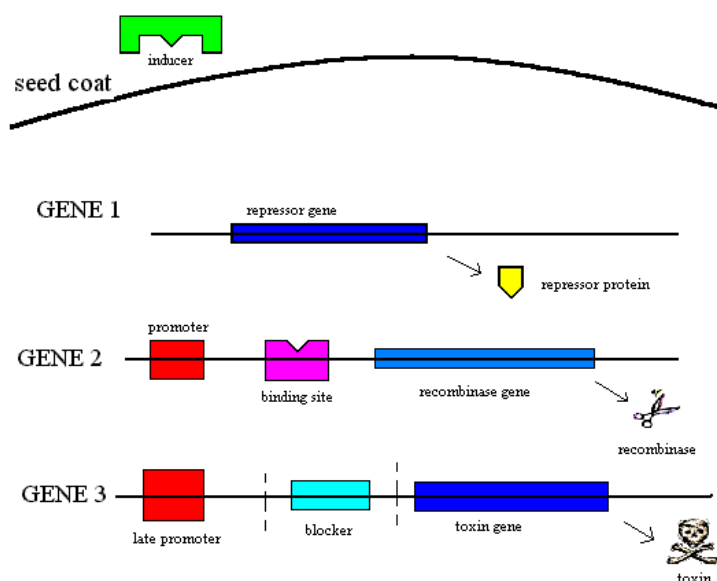


Figure 3.2 'Terminator' technology consists of three genes

Gene I is a repressor gene that produces a repressor protein that interacts with a binding site near Gene II. Gene II is a recombine gene that is controlled by a promoter. Between the gene and the promoter is a binding site for the repressor from Gene I. The recombine gene produces a recombine protein that is an enzyme and snips out pieces of DNA. Gene III produces a toxin that is lethal to embryos. The gene is controlled by a late promoter, which is active only during the late stage of seed development when the embryo is developing. Between the late promoter and the toxin gene is a piece of DNA called a blocker, which interferes with the ability of the promoter to turn on the toxin gene. **INDUCER:** The inducer is a chemical applied to the seed by the seed company that will initiate the terminator gene interactions.

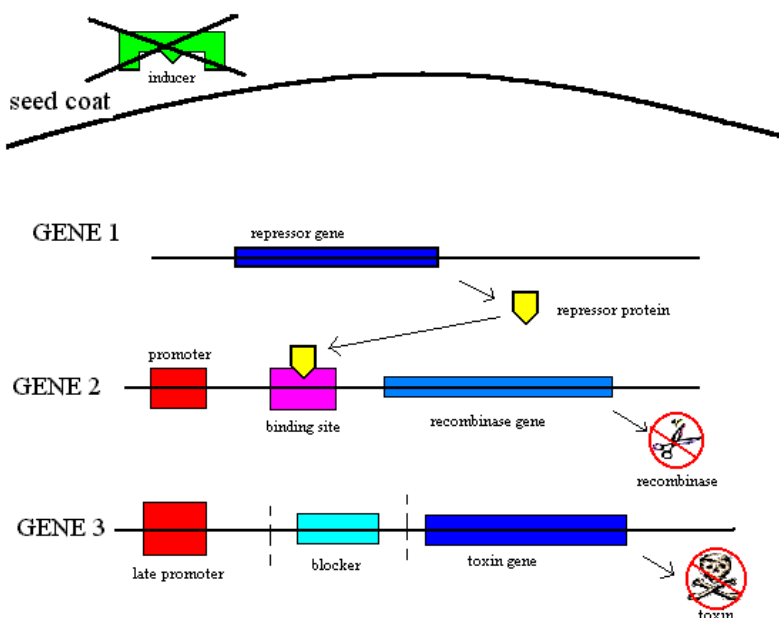


Figure 3.3. 'Terminator technology': production of viable seeds

If the seed company does not want to initiate the terminator genes, it will not apply the inducer. This allows the repressor protein to bind to the binding site on Gene II, preventing the production of recombine. In the absence of recombine, the blocker on Gene III is not snipped out, and the toxin is not produced. This allows the seed company to raise enough seed to sell to farmers.

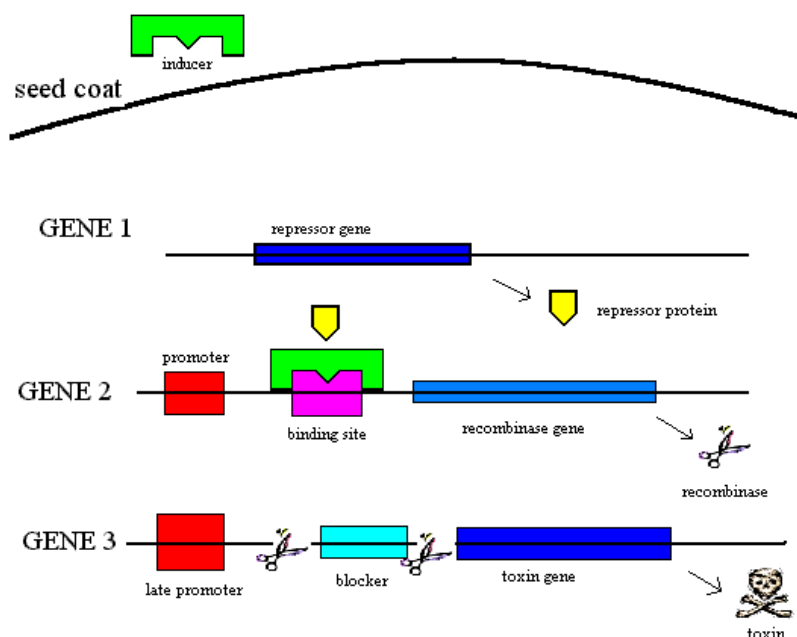


Figure 3.4 'Terminator technology': inhibition of seed viability

Before the seed company sells the seed to the farmers, it applies the inducer. The inducer blocks the binding site on Gene II preventing the repressor protein to bind. Gene II then produces recombinase which snips out the blocker on Gene III. With the blocker removed, the late promoter is able to turn on production of the toxin gene late in the season.

Similar strategies within the same transgene construct have been proposed, using alternative stimuli, such as temperature or osmotic shock to regulate the mechanism, and by using abnormal levels of plant hormones as the cytotoxic element leading to cell destruction (Daniell 2002).

In a related study, Schernthaner *et al.* (2003) propose a repressible seed lethal system whereby viable seeds are produced by the GM crop plant, when crossed (or selfed) by another GM crop plant in the same field, but non-viable seed are produced when the crop plant is crossed (in either direction) with non-GM plants. This method also uses seed lethal genes with a repressor element, where the seed lethal (SL) element is tightly linked to the desired novel trait. The repressor element (R) is crossed into the seed during hybrid seed production; thus the planted seed contains both the SL and R element. The presence of the repressor silences the seed lethality gene and thus the seed generated by the crop is fully viable. Therefore, the seed that result from the selfing of the crop are able to germinate.

Should out-crossing or in-crossing occur between the SL/R crop plant and a plant lacking the repressor element R, the two are separated. In the absence of the R element, the seed lethality gene is activated in the seed embryo and thus any seed containing the novel trait will not germinate (Schernthaler *et al.* 2003).

Separation of the SL and R elements does enable the escape of the repressor element into other crops or wild populations. Contamination of conventional crops with a transgene of any kind presents issues with regard to their non-GM status. The escape of the repressor element into wild or weedy populations offers the possibility for escape of the functioning SL transgene should a wild plant carrying the repressor element be grown in proximity to a crop carrying the same construct in subsequent years. In addition, seed escape during harvest would enable the GM crop to become feral as any spilt seed will be viable and will express the novel trait.

Gressel and Al-Ahmad (2005a) claim that this particular system is technically impractical as the seed lethal trait and the repressor element must be simultaneously inserted at the same locus on homologous chromosomes in the hybrid seed and site-specific insertion of transgenes has not yet been achieved in higher plants. In addition, the hemizygous seed lethal parent would not be able to produce viable seeds, and of the seed produced only 25% would contain both the seed lethality and repressor element, with the remaining 75% containing only one element (Gressel & Al-Ahmad 2005a). Such a system, therefore, requires additional development in order to become a reliable containment methodology.

All relevant national and international agencies have considered the potential impact of the various GURT technologies. For example, In 2000, the United Nations' Convention on Biological Diversity (CBD) (<http://www.biodiv.org/programmes/areas/agro/gurts.asp>) recommended that *"in the current absence of reliable data on genetic use restriction technologies, without which there is an inadequate basis on which to assess their potential risks, and in accordance with the precautionary approach, products incorporating such technologies should not be approved by Parties for field testing until appropriate scientific data can justify such testing, and for commercial use until appropriate, authorized and strictly controlled scientific assessments with regard to, inter alia, their ecological and socio-economic impacts*

and any adverse effects for biological diversity, food security and human health have been carried out in a transparent manner and the conditions for their safe and beneficial use validated".

Apart from Monsanto and Delta & Pine Land, other companies having patents or applications relating to GURT technology include Syngenta, DuPont, and BASF. As regards the present state of GURTs, the strength of public opposition has led to seed companies withdrawing such systems from commercial development. Analysis of the relevant databases suggests that there are no confirmed field trials of these technologies (if defined to mean production of sterile seed), though presumably research programmes may be continuing in glass houses etc. However, it should be noted that there have been field tests of various chemically switchable promoters (Section 3.2.3.2), which may be one component of a GURT system. Although the various mechanisms are considered too complex and unreliable by some critics, others suggest the systems may represent a useful research tool and more robust methods are being developed (Budd 2005). Since such technology could be adopted in one form or another as a gene containment strategy it could be particularly useful in phytoremediation and in bio-pharming (see Section 4 below) where replanting saved seeds is not a priority, in situations where the seeds are not intended for human and animal consumption, and where environmental dissemination is to be avoided. In a recent review of various genetic containment methods (Lee & Natesan 2006) the authors conclude "Because they are generally lethal in hybrids, GURTs can have an important role in controlling gene flow and introgression. If particular caution is required, multiple strategies can be used to manage the concerns surrounding transgenes with the greatest potential for environmental or human health impacts."

The present Defra position on GURTs is available at:-

<http://www.defra.gov.uk/environment/gm/eu/gurts-0602.htm>

3.2.3.5 Apomixis

The use of apomixis (i.e. the production of fruit or seed without the need for fertilisation and pollination) is a potentially important tool for the containment of

transgenes. In addition, as seed is produced without pollination the maternal genotype becomes fixed, thus enabling the fixation of uniform and superior generations (Daniell 2002; Anon 2004). However, the genetic regulation of apomixis is complex and is most likely polygenic. As such, it is likely that a complex gene construct would be required to induce genetically engineered apomictic crop production (Gleba *et al.* 2004). The transfer of apomixis to crop species via conventional methods of crossing and backcrossing over a forty-year period has so far failed (Perotti *et al.* 2004) and current understanding of apomixis is not at a point where it can be engineered.

Some recent advances have been made however, as Spielman *et al.* (2003) suggest that part of the difficulty in transferring apomixis to other species is due to the nature of endosperm development. In seeds produced from sexual reproduction, the endosperm is derived from two maternal cells and one paternal cell. In apomicts, the endosperm is entirely maternal. Spielman *et al.* (2003) suggest that modification of DNA methylation could alter the imprinting system that determines endosperm development and induce endosperm development in the absence of a male gamete.

Apomixis is considered by some to have a limited application in terms of containment of GM (Gleba *et al.* 2004). In particular, there are several concerns regarding the leakiness of apomictic systems. Absolute apomicts are rare in nature and many apomicts retain a low to moderate level of sexual reproduction, and in some cases moderate to high levels of pollen fertility are common. Some apomicts require pollination as a stimulus to seed formation although gamete fusion does not take place (Anon 2004). If viable pollen containing engineered apomixis genes is produced by the crop then the transgenes are not contained. The escape of apomictic genes into wild populations of plant species not naturally carrying such genes could lead to considerable damage to the natural population structure. Due to the physiological 'cost of sex' by the production of flowers, apomicts will always replace out-crossing organisms when all else is equal (Anon 2004). Thus, transgenes could spread quickly throughout a population leading to the apomicts becoming invasive and the natural population becoming extinct by swamping (Anon 2004).

3.2.3.6 Cleistogamy

Cleistogamous plants have flowers that do not open. Self-pollination and fertilisation takes place within the closed bud and there is no release of pollen or exposure of the gynoecium to out-crossed pollen. According to Lu (2003), transgene containment by the use of cleistogamy appears to be innovative for some crop species, but it is not practical for all crop species. Many crops such as maize, common buckwheat, cassava, and most cucurbits are allogamous, and it is extremely difficult to create cleistogamous plants for these crops. Even in the case of autogamous crops, the manipulation of cleistogamy may alter floral structures and natural flowering habit of some species. Such alteration might affect yield of those crops whose grains/seeds are the part harvested.

As with apomixis, no wild plant species produce entirely cleistogamous flowers and there will be a need to ensure leakiness does not develop. For example, in rice lines that exhibit cleistogamy, gene flow occurs between feral and cultivated forms (Daniell 2002). Despite the potential benefits, cleistogamy is not being developed as a potential containment strategy (Anon 2004) and identification of genes that could be used to engineer cleistogamy remains remote (Daniell 2002).

3.2.3.7 Transgene Mitigation (TM)

This concept has been championed by Jonathan Gressel who has published extensively in the last few years (Al-Ahmad & Gressel 2005, 2006; Al-Ahmad *et al.*, 2005, 2006; Gressel 1999; Gressel & Al-Ahmad 2004, 2005a). It is also the subject of a patent application (US 040172678).

It is argued that most transgene containment mechanisms are unidirectional, as they will only prevent gene flow in one direction and as such are not absolute (Gressel and Al-Ahmad 2005a). As these mechanisms are leaky there is a need to add additional genes to mitigate against the spread of transgenic hybrids in wild populations by expressing traits that render hybrids unable to compete against wild plants, but that will not reduce the performance of the crop plants in the field. Thus,

the transgene-of-interest conferring the desired trait could be part of a tandem construct containing a second gene that is beneficial, or neutral, under agricultural conditions, but disadvantageous in the wild. Examples include genes that prevent seed-shatter or secondary dormancy or that induce dwarfism. Proof of principle was obtained using a cassette consisting of a semi-dominant gibberellic-acid-insensitive gene that causes dwarfing, and a model desired-trait for herbicide resistance. Greenhouse experiments with tobacco and oilseed rape showed that plants containing the cassette were unable to compete with normal plants when sown in close spacing in the absence of herbicide treatment to mimic competition in the wild. In the absence of competition, then both the tobacco and oilseed rape plants produced a good crop with reduced stem height, yet with increased leaf and flower production (Al-Ahmad *et al.* 2004, Al-Ahmad & Gressel 2006). For phyto-remediation purposes, this technique is inapplicable in its present form, since competition with wild plants may be a desired trait.

According to Lu (2003), for TM to be successful, the inserted gene cassettes must comprise tightly linked tandem constructs of the TM trait and the desired trait of interest such that their separation during meiosis is extremely rare. However, there are some concerns with TM as a method of transgene containment. First, this technology does not address the problem of transgene escape from a GM crop to non-GM crop varieties. In addition, transgene escape is allowed to occur to weedy or wild species through gene flow, even though any resulting hybrids are engineered not to persist in the population. The destiny and long-term consequences of the mitigation genes in crop and weedy/wild populations are as yet unpredictable as the traits used to compromise fitness such as seed dormancy, seed shattering, and delayed seed ripening, are usually controlled by dominant alleles. Second, the organization of tandem constructs with tightly linked genes will require considerable efforts of multigene engineering. Envisaging future attempts to transfer multiple genes with different functions into one crop variety in later generations of transgenic biotechnology make the constructs particularly difficult and as such, TM technology is still at the developmental stage.

3.2.3.8 Recoverable Block of Function

A system of transgene containment has been developed to include a two-factor 'blocking sequence' and a 'recovering sequence' within the same transgene construct as the transgene of interest. This 'recoverable block of function' system developed by Kuvshinov (2001a,b; 2004; US Patent Applications 050039229, 040107457, 040025200) inseparably links the blocking sequence to the transgene of interest by inserting it into an artificial intron within the transgene sequence. The expression of the blocking sequence may be used to confer cell death or to prevent sexual reproduction. The recovering sequence is induced by an external stimulus such as chemical application or heat shock and expression results in the expression of the normal phenotype (Gleba *et al.* 2004).

This system has been demonstrated in tobacco where the ribonuclease barnase was used as the blocking sequence and is expressed from a sulphhydryl endopeptidase promoter, active at the time of seedpod development, thus preventing seed germination. The 'antidote' to barnase is the expression of the barstar gene, which is placed under the control of a heat-shock promoter. Prolonged heating of the developing seeds to 40°C in a greenhouse results in barstar production and removal of barnase expression, thus permitting correct seed development and germination. Any seed formed from hybridisation between wild relatives and the GM crop will be unable to germinate due to the expression of the blocking construct as they will not encounter the prolonged high temperatures necessary for removal of the barnase block, and so would fail to germinate (Kuvshinov *et al.* 2001a).

This system has been tested experimentally, but there is no record of this system having been trialled on a field scale.

3.2.3.9 Inteins

Protein splicing elements, known as inteins are fragments of a protein sequence that are 'spliced out' or excised during the post-translational process to form a new mature protein (Zeidler *et al.* 2004; Perler 2005; Xu & Evans 2005). The action of

inteins makes them natural protein engineering elements, which can be harnessed in a number of ways, such as the synthesis and purification of proteins.

Inteins can be used to reconstitute a functional protein from fragments of an inactive protein. This function can be exploited in order potentially to limit transgene spread by reducing the possibility that a transgene expressing an active protein can escape into wild populations or non-GM crops (Chen *et al.* 2001; Sun *et al.* 2001; Chin *et al.* 2003). The transgene is divided into two sections, each of which expresses an inactive, truncated protein. These gene fragments can be inserted into different locations in the plant cell, such as the nuclear and plastid genomes. Expression of the genes occurs separately within the nuclear and plastid genomes, a localisation signal from the plastid is able to target the gene product in the nucleus via the cytoplasm, and the nuclear expressed gene product is trans-located to the chloroplast. Within the chloroplast, the two products are *trans*-spliced via intein mediation to generate a full-length transgene product (Evans *et al.* 2005).

By separating the two gene fragments between the nuclear and chloroplast genome, pollen cells from such a plant will encode only a truncated, inactive transgene product. Furthermore, should some plastid DNA escape it also encodes only a portion of the inserted protein. Additional security could be developed for some crops by the insertion of the nuclear transgene fragment into a genome that is not compatible with weedy relatives. For example, crops such as oilseed rape and wheat have multiple genomes as they are derived from hybrids of different wild relatives. Specifically, wheat is comprised of three genomes designated A, B and D (Hedge & Waines 2004), whereas its wild relative, jointed goatgrass (*Aegilops cylindrica*) has only two, C and D. The location of the nuclear encoded fragment on the A or B genome of wheat would therefore significantly reduce the risk of transgene spread to wild species, but would not prevent crop-to-crop movement.

Intein mediated *trans*-splicing of proteins has been demonstrated in *Arabidopsis* (Yang *et al.* 2003) and in tobacco (Chin *et al.* 2003) using marker-based expression systems and herbicide resistance genes. Proof of concept of the use of inteins in transgene containment (Evans *et al.* 2005; Khan *et al.* 2005a; Zhao *et al.* 2004) and molecular pharming (Morassutti *et al.* 2002) has been achieved in a small number of

experimental studies (Chen *et al.* 2001; Sun *et al.* 2001; Chin *et al.* 2003; Yang *et al.* 2003). There is also a series of patents relating to this methodology (US Patent Applications 040172688, 040096938, 030167533).

Despite reported successes of this methodology, the *trans*-splicing of the proteins within the plant cell requires optimal temperature and pH conditions in the laboratory to ensure the proteins fold into the correct structure, making the process still experimental and not yet sufficiently robust to be performed in the field. To date, no field trial application has been made for plants with the inserted intein SspDnaE.

Regulatory issues regarding this technology must still consider that the partial construct of 'engineered, non-native' DNA may still enter the genepool of wild species and non-GM crops. The genetic engineering of crop species with the *trans*-splicing technique may offer an additional level of protection for transgene containment but is unlikely to prevent transgene escape in isolation (Evans *et al.* 2005).

3.2.3.10 Auxotrophy

Auxotrophy is a method adopted in microbial research as a means of containing fungal, bacterial, viral strains and cell cultures within the laboratory environment and of preventing sample contamination. The organism is first transformed to become dependent upon a compound that is made exogenously available, and as a consequence it is then unable to survive or develop normally in the absence of that compound, and untransformed organisms cannot survive in the presence of the compound.

This has been demonstrated experimentally in plants with manipulation of the methionine synthesis pathway in *Nicotiana plumbaginifolia* and *Arabidopsis* (Kim & Leustek 2000; Frankard *et al.* 2002). Interruption of the methionine biosynthesis pathway by the antisense expression of the *CGS* gene in *Arabidopsis* resulted in severe growth stunting, morphological abnormalities and an inability to flower. These traits were all reversed by the exogenous supply of methionine metabolites (Kim & Leustek 2000).

Arabidopsis auxotrophs dependent upon biotin (Patton *et al.* 1996) and auxin (Hobbie & Estelle 1994) have also been developed. Similarly, tobacco plants have been transformed with the *cys1* gene from wheat to be resistant to hydrogen sulphide gas (Youssefian *et al.* 1993).

Manipulation of the environment around plants on a large scale may not be appropriate, and as such, this concept may have practical value only for aquatic plants such as unicellular algae. The algae would be dependent on the presence of a substance in the water of the growth tank that would not be readily available in a natural environment. In such a way, transgenic algae could be prevented from establishing in natural bodies of water.

3.2.3.11 Transgene excision

There is a series of genes that can be inserted into the plant genome and thereby encode enzymes that are able to insert, excise, invert or translocate fragments of DNA. Such recombinases act on specific sites and can be used as part of a genetic use restriction ('terminator') system (see section 3.2.3.4) to activate constructs or can be used to remove or deactivate a construct. The enzymes target specific DNA sequences that can be engineered to flank the transgene construct or a disabling intron inserted within the transgene sequence.

Removal of a transgene can be achieved at different developmental stages or in specific tissues depending on need (Hare & Chua 2002; Ow 2005; Wang *et al.* 2005a). For example, recombinase genes may be engineered to excise transgenic DNA during pollen development such that male gametes do not carry the transgene. This offers an alternative strategy for self-pollinating crops where male sterility is not a viable option (Ow 2005). Alternatively, a fruit specific inducer may be used to eliminate the transgene cassette from the fruit thereby producing non-transgenic fruit from transgenic plants. Such technology would address issues of seed escape during harvest and issues of food safety (Keenan & Stemmer 2002).

Site-specific recombination is a flexible system and can also be applied in different ways. For example, it can be used as an inducible promoter to activate a marker gene by expressing recombinase to remove an insert from within the gene to allow normal function (Hoa *et al.* 2002).

The *cre* recombinase based system has been demonstrated to be highly site-specific (Gilbertson 2003) but some areas of concern remain, particularly with the reliability of the technology to function absolutely. The use of chemical inducers to activate the recombinase may not be completely effective due to incomplete or insufficient application of the chemical. There is an additional possibility that the recognition site of the recombinase will remain intact within the genome and will be present as non-native DNA. As such, these non-native sequences may be passed into wild or weedy populations of related species or into non-GM crops, thereby potentially jeopardising its non-GM status (Keenan & Stemmer 2002). In addition, the fate of the DNA fragments once they have been excised from the strand of genomic DNA requires investigation. It had been assumed that the nucleotides would be degraded and assimilated in the cell. Following *cre* mediated excision of transgenes in wheat, deletion products have been detected inside the cell. These had formed into an extra-chromosomal circular molecule that was passed into subsequent generations (Srivastava & Ow 2003).

Transgene excision has clear potential as a containment strategy, but remains in the developmental stage. Further research into the function and persistence of deletion products are required to ensure the absolute reliability of recombinase on a large scale and to address the problems of the persistence of non-native DNA.

3.2.4 Summary of containment strategies

The current range of transgene containment strategies, based on available data, is summarised in Table 3.4.

Table 3.4 Summary of GM containment strategies
(Based on Daniell 2002; Anon 2004; Gleba *et al.* 2004).

Technique	Advantages	Disadvantages	Status
Physical separation	Growing transgenic plants expressing valuable industrial compounds are easier to monitor in physically contained areas Removal of external factors of growth into a completely controlled environment	High costs incurred from growing plants indoors or under ground Regulation of large scale underground facilities unclear	Under development for field crops Contained growth of non-GM algae is established
Natural genetic containment	Choice of an organism not used in the human food chain for bio-pharming applications could prevent the compound entering the human food chain Expression of transgene product in the leaves may negate need for flowering	Selection of a new organism may set a project back in time and increase costs Cultivation, harvesting and processing may not be established for new organisms	Expression of bio-pharming products in non-conventional crop species under development (see section 4)
Plastid transformation	Prevents escape via out-crossing Well developed High levels of transgene expression Well suited to bio-pharming	Does not prevent escape via wild-to-crop pollination Not all plants have 100% maternal inheritance Many desirable traits cannot be produced by proteins in the chloroplast	Successful technique in tobacco Demonstrated in potato, tomato, petunia, cotton, carrot, soybean Field trials of tobacco in the US expressing pharmaceutical proteins
Conditional lethality	Control of lethality may be timed to prevent flowering or the development of reproductive organs	Incomplete expression may occur if the application of the chemical inducer is insufficient or fails to penetrate plant tissues	Not yet demonstrated in the field
Inducible promoters	Gene only activated when necessary	Confines the trait but not the transgene Not applicable to traits required throughout the life of the plant Incomplete expression may occur if the application of the chemical inducer is insufficient or fails to penetrate plant tissues	Not yet demonstrated in transgenic crops

Table 3.4 *continued*

Technique	Advantages	Disadvantages	Status
Engineered male sterility	Prevents out-crossing to wild and to non-GM crop plants Well developed in a wide range of crop plants	Seed crops require additional pollen source Potential for volunteer seed dispersal Male sterility often leaky Recombination of chromosomes during meiosis could separate transgene from sterility system	Barnase based male sterility demonstrated in tobacco, rice, maize, alfalfa, oilseed rape, tomato, wheat, citrus and birch 121 field trials in the US of maize, oilseed rape and <i>Brassica oleracea</i> crops with barnase based male sterility 19 field trials in Europe of crops with barnase based male sterility There are 2 lines of oilseed rape, 2 of maize and 1 of chicory commercially available with barnase based male sterility Male sterile oilseed rape is widely grown in Canada and is pending release in the EU under directive 2001/18.
The 'terminator' concept and seed lethality	Prevents transgene escape via out-crossing and volunteer seed dispersal	Recombination of chromosomes during meiosis could separate transgene from sterility system Not suitable for crops where seed is saved Negative public perception	'terminator' technologies withdrawn from commercial development and not been demonstrated in the field
Apomixis	Prevents transgene escape via out-crossing and volunteer seed dispersal	Genes controlling apomixis not yet identified but likely to involve a complex gene construct Likely to be leaky GM apomicts likely to be invasive Does not prevent seed dispersal	Genes controlling apomixis not yet identified Not yet demonstrated in transgenic crops
Cleistogamy	Prevents transgene escape via out-crossing and volunteer seed dispersal	Genes controlling floral development not yet identified Likely to be leaky Seed escape may still lead to volunteers Not suitable for all crops Does not prevent seed dispersal	Very little development as a containment strategy Genes controlling cleistogamy not yet identified Not yet demonstrated in transgenic crops
Transgene mitigation	Prevents introgression of transgenes into wild or weedy populations Addresses crossing of GM crop in both directions	Does not prevent gene flow from GM crops to non-GM crops May be harmful to natural populations of wild relatives Allows formation of transgenic F ₁ hybrids Depends on weed competition to succeed Recombination of chromosomes during meiosis could separate transgene from mitigation system	Demonstrated experimentally in tobacco and oilseed rape Requires further development
Recoverable block of function	Transgene and containment system are inseparable during chromosome recombination Can be engineered to control pollen or seed sterility	Incomplete expression may occur if the application of the chemical inducer is insufficient or fails to penetrate plant tissues	Demonstrated experimentally in tobacco

Table 3.4 *continued*

Technique	Advantages	Disadvantages	Status
Inteins	Prevents escape of a complete, functioning transgene	Allows escape of non-native but non-functioning DNA May 'contaminate' 'non-GM' crops Function of the recombinase requires optimal conditions that may not be obtainable in the field	Demonstrated experimentally in tobacco and <i>Arabidopsis</i>
Auxotrophy	Prevents survival of transgenic plant outside controlled environment	Does not prevent formation of F1 hybrids Recombination of chromosomes during meiosis could separate transgene from mitigation system High costs on a large scale	Not well developed as a containment mechanism Demonstrated experimentally in <i>Nicotiana plumbaginifolia</i> and <i>Arabidopsis</i>
Transgene excision	May be engineered to produce non-transgenic seed/fruit from transgenic plants	Requires complete expression of the recombinase Recombinase excision site will remain as non-native DNA Deletion products may remain intact within the cell and may be passed on to the next generation Not applicable to traits expressed in seeds	Requires further development

3.3 Containment issues relating specifically to trees

Wood is still a crucial source of fuel for both heating and cooking for many and demand for wood continues to rise with increasing population size and economic development (Campbell *et al.* 2003). Indeed, current projections estimate 25% of the global forest resources must be logged to meet demand by 2050 (Bradshaw & Strauss 2001). There are also additional demands made upon forest resources as a method to sequester carbon dioxide and reduce the accumulation of greenhouse gases and as the conservation movement calls for the preservation of forest ecosystems (Campbell *et al.* 2003).

There are intensive and increasing pressures therefore on native woodlands (Bradshaw & Strauss 2001). Within Europe, changes to the EU Common Agricultural Policy are likely to result in the planting of low grade agricultural land with trees. Such changes are aimed at relieving these pressures in regions where they are most acute, namely in Central and Eastern Europe (Gartland *et al.* 2002). and there is an associated need to increase the productivity of existing plantations.

Conventional tree breeding programs are hampered by the protracted life cycle of long-lived, slow to mature trees. In addition, as trees are mostly out-crossing and heterozygous in nature, there are many recessive deleterious alleles retained via conventional breeding and desirable alleles are difficult to fix in true breeding lines (Campbell *et al.* 2003).

The development of genetically modified forest trees offers alternative sources of wood, fuel and fibre and presents new opportunities for increasing productivity and product quality (Bradshaw & Strauss 2001).

3.3.1 Concerns about GM forest trees

There are several issues that relate specifically to the containment of transgenes from trees and these are: the heightened risk of gene flow, long-term transgene stability, the risk of invasiveness, and effects on non-target organisms (Fladung 2002).

Commercial forest trees are essentially undomesticated, thus making the escape of transgenes more probable (Bradshaw & Strauss 2001; DiFazio *et al.* 2004; van Frankenhuyzen & Beardmore 2004). Most commercially grown forest trees are cultivated in proximity to cross-compatible wild relatives and many exotic plantation species have escaped cultivation and formed feral populations with which cultivated exotics can cross (Campbell *et al.* 2003; DiFazio *et al.* 2004; van Frankenhuyzen & Beardmore 2004). Gene flow via pollen and seed is potentially much greater for tree species than for conventional field crops for several reasons. Trees are out-breeders and produce copious amounts of wind borne pollen that can travel great distances (Anon 2004; Burczyk *et al.* 2004; DiFazio *et al.* 2004; Slavov *et al.* 2005). In addition, trees are long lived and reproduce over many decades (Gartland *et al.* 2002). As trees get taller, seed and pollen can escape further and as trees become older they produce more pollen and seed. Although 99% of pollen and seeds are dispersed locally, approximately 1% of pollen and seed are vertically uplifted above the forest canopy by air currents and may be transported kilometres from the source tree (Gartland *et al.* 2002; Williams 2005).

The escape of any transgenic trait via gene flow or seed escape has the potential to lead to the development of invasives resulting in unwanted change and effects on the flora and fauna via conferred advantage. The escape of insect resistance transgenes to wild populations could affect populations of indigenous organisms, compounded by the fact that trees are long lived (Anon 2004). In addition, the use of insect resistant genotypes in plantations will increase selection pressure on insects to develop resistance, again made more acute by the longevity of the crop (van Frankenhuyzen & Beardmore 2004). Escape of herbicide resistance traits is only

thought to confer advantage in habitats where herbicide use is extensive. Conferred advantage is difficult to assess over long periods and only feasible via models based on short-term studies. Not surprisingly, these models suggest that genes encoding insect and disease resistance are more likely to introgress than genes encoding herbicide tolerance (van Frankenhuyzen & Beardmore 2004).

The development of GM invasive trees is not considered to be an issue by some as GM native trees should be no more invasive than non-GM trees as they are generally poor competitors with existing weeds (Gartland *et al.* 2002). However, there are considerable problems with introduced forest species becoming invasive (van Frankenhuyzen & Beardmore 2004) and should these become transgenic the problem would be compounded. Therefore, the cultivation of GM exotics requires stricter regulation and containment than native trees (DiFazio *et al.* 2004).

Amongst the range of GM projects in trees, transgenes altering the biosynthesis of lignin are being developed to reduce the costs and chemical input required to remove lignin prior to pulping and paper manufacture. As lignin provides the structural strength of wood, any reduction in lignin may lead to a reduction in tree fitness and ability to survive cold temperatures (Anon 2004; van Frankenhuyzen & Beardmore 2004). Lignin also reduces the digestibility of plant material acting as a natural defence against insects and pathogens. Long-term effects of lignin reduction are not known, but the increase in susceptibility to infection may undermine the commercial value of such traits (van Frankenhuyzen & Beardmore 2004). The escape of these genes is likely to be selected against in the wild, as it would appear to be disadvantageous (Anon 2004). There are also suggestions that altering the lignin content of trees may affect soil structure and fertility as the decomposition of organic matter is likely to be accelerated. This in turn may affect nutrient cycling and micro-organisms in the soil, and the ability of the tree roots to form symbiotic interactions with mycorrhizae (van Frankenhuyzen & Beardmore 2004). Trees are the dominant species when present in ecosystems and directly and indirectly support a large composition of organisms. Thus, any change in the physiology of the tree has the potential to have a large effect on the ecosystem surrounding it (Anon 2004; DiFazio *et al.* 2004).

In addition, concerns exist over the long-term stability of transgenes in trees as they are longer lived than field crops (Anon 2004). Trees experience repeated cold induced dormancy phases and will encounter a range of environmental conditions during a normal rotation. Regulations of controlled release of GM trees in field trials often do not allow field trials to continue beyond the juvenile phase and the onset of the reproductive phase and as such, the stability of transgene expression is not tested over a full rotation (van Frankenhuyzen & Beardmore 2004). More research is required to investigate the effect of the site of transgene insertion and its effects of gene expression and stability (Gartland *et al.* 2002).

The need for a containment strategy for transgenic trees needs to be assessed on a case by case basis and further field trials are required to fully investigate many of the concerns raised (Ellis *et al.* 2001). Indeed, the use of transgenic disease resistance to revive populations of native trees devastated by the introduction of non-native pathogens would not require a containment strategy (Anon 2004).

3.3.2 Containment options

The induction of transgenic sterility would appear to address most concerns with GM trees. Not only would this eliminate the concerns of gene flow via pollen (Williams & Davis 2005), such a strategy would prevent production of pollen allergens. In addition, it has the potential to lessen the slowing of growth associated with maturation and therefore to increase productivity (Campbell *et al.* 2003; Meilan *et al.* 2004).

Genes controlling flowering appear to be conserved among angiosperms and even extend to some gymnosperms (Campbell *et al.* 2003) and transgenic sterility can be induced in a number of ways (Ellis *et al.* 2001; Anon 2004; Meilan *et al.* 2004). Floral tissues can be prevented from developing fully via cell ablation. Cytotoxic elements linked to floral specific promoters can be used to induce the early death of cells e.g. the ribonuclease barnase. Dominant negative mutations can be engineered to encode proteins that suppress the activity of the native proteins expressed during floral organ development. Post-transcriptional gene silencing can be induced by RNA

interference (RNAi). This latter technique uses the introduction of a transgene that contains an inverted repeat region of a sequence corresponding to part of a transcribed region of the endogenous gene targeted for silencing. The repeat region interferes with the protein coding process and floral organs will not develop fully (Ellis *et al.* 2001; Meilan *et al.* 2004).

Stability of sterility traits is again an issue as some tree rotations last more than 40 years (Anon 2004) and occasional reversions should be expected (Meilan *et al.* 2004; van Frankenhuyzen & Beardmore 2004). Field trialling of sterility traits requires taking trees through to maturity, and this will necessitate additional containment measures such as biological buffer zones and physical isolation which will also increase the economic costs of the trial (Campbell *et al.* 2003).

Complete sterility may not be required in all cases and the absence of flowers and fruit from forest plantations will further reduce any biodiversity in the area. In some instances, it may be beneficial to wildlife for forest trees to produce intact flowers and fruit (Meilan *et al.* 2004).

Several other options for containment have potential but require further development. Triploid poplars have been used in the past and have a high level of innate sterility. Methods of tissue specific expression may offer alternative containment strategies as leaf and vasculature specific promoters have been identified in poplar and spruce (Gray-Mitsumune *et al.* 1999; No *et al.* 2000) but tissue specific expression has not yet been demonstrated (Anon 2004).

Methods of transgene containment for GM trees require further development and trialling. In particular, monitoring of field trials over long periods remains an issue and the need for large areas further increases development costs (Anon 2004). The long time lag between research, development, trialling and demonstrated commercial potential is a barrier to the continuation of GM development in forest trees (van Frankenhuyzen & Beardmore 2004). Indeed the benefits of insect resistance and herbicide tolerance may not provide sufficient return on the investment to make commercial release viable (Valenzuela & Strauss 2005). Levels of industrial interest in the development of GM forest species are low at present, as researchers in some

parts of the world turn away from GM technology due to concerns over public opinion and the costs of development and trialling. However, some workers express concerns over the difficulties in the development of GM trees and the missed opportunities for their use in phytoremediation (Peuke & Rennenberg 2005) and disease resistance and in reducing the economic pressure on native forests (Valenzuela & Strauss 2005).

Research into the commercial development of lines of GM forest and fruit trees remains active and field trials are still taking place. At present 280 applications have been made to trial GM forestry trees and 83 to trial GM fruit trees (Table 2.2). In the United States, Cornell University have successfully developed a GM line of ringspot virus resistant papaya that has been deregulated by APHIS for commercial planting (Appendix V). In addition, The University of Florida have developed another line of GM papaya resistant to ringspot virus, and ARS have developed a GM variety of plum resistant to plum pox both of which have applications for deregulation pending under APHIS (Appendix VI).

3.3.3 Summary of containment issues relating to trees

Gene flow from GM trees is a concern because:

- Out-breeding, wind borne pollen capable of travelling long distances
- Long-lived perennial
- Forestry species are undomesticated genotypes
 - Cultivated close to cross-compatible wild relatives
 - Exotic species often escaped cultivated and feral populations exist
- Dominant species in their ecological niche
 - Interact with many organisms

Containment options:

- Male sterility
- Ploidy levels
- Tissue specific expression

Problems:

- Long term transgene stability over lifetime of organism
- Length of trial often restricted to juvenile phase of growth to prevent flowering
 - Trialling long term stability restricted
 - Trialling of sterility restricted

3.4 Containment issues relating specifically to grasses

Improvement of grasses via genetic modification offers a variety of benefits from improved digestibility for forage grasses to increased manageability of turf grasses. The large turf and amenity grass market has focussed development of transgenic grasses towards the turf grass species, widely used in landscaping and golf courses. Beneficial traits such as herbicide and disease resistance will maintain species stands and reduce chemical input required to manage infection and infestation by other species. Improved drought and stress tolerance in grasses will reduce the need for irrigation and increase the use potential of marginal land. In addition, the induction of insect and pest resistance traits will reduce the need for pesticide applications. Horticultural qualities can be engineered to change pigmentation or confer a stay-green appearance (Luo *et al.* 2004). Indeed, Monsanto are developing a GM turf grass that will require less mowing (Anon 2004).

3.4.1 Concerns of GM grasses

Containment of GM grasses presents several challenges. Grasses are perennial, inherently out-crossing, capable of hybridisation with related, weedy species, and able to propagate themselves vegetatively. The perenniality of grasses means that any spontaneous hybrids occurring between species will persist and flower from season to season, increasing opportunities for further backcrossing and introgression. Most grass species are self-incompatible and out-crossed by wind borne pollen. Grass pollen is known to travel great distances and workers have taken the opportunity to assess transgene movement during GM field trials of grasses in the US. Wang *et al.* (2004b) examined pollen mediated transgene flow in *Festuca arundinacea* and found transgenic traits in populations of grasses up to 150m from the crop, but no further than 200m. Watrud *et al.* (2004) analysed gene flow from a transgenic crop of *Agrostis stolonifera* and found that most gene escape occurred within 2 Km in the direction of the prevailing winds. Some pollen was found to travel much further with the maximum gene flow distances measured being 21 Km from the field site (Watrud *et al.* 2004). Gene flow and introgression

between non-transgenic *Sorghum bicolor* (cultivated sorghum) and *S. halepense* (Johnson grass) has been assessed using a range of crop specific markers and found that gene flow occurs between sympatric populations, with up to 32% of individuals in a population containing DNA of sorghum origin (Morrell *et al.* 2005). In addition, populations of Johnson grass with no recent exposure to sorghum were found to contain sorghum alleles; this suggests that sorghum pollen had travelled long distances and that the introduced alleles had become stabilised in the population (Morrell *et al.* 2005).

Grasses can also reproduce by vegetative means via rhizomes and stolons and these in turn be moved around by agricultural machinery. In addition, seed can be dispersed by birds and mammals feeding and foraging in the grass (Anon 2004).

3.4.2 Containment options

Most of the containment strategies discussed in Section 3 of this report may be applied to grasses. Development has focussed on male sterility as pollen mediated gene flow over long distances causes most concern. Male sterility via cell ablation has been demonstrated in several grass species including *Agrostis stolonifera* (Luo *et al.* 2004; US Patent Application 050235379). Interruption of the pollen development pathway will also reduce the amount of pollen in the air and be of benefit to allergy sufferers.

To date there have been just over 200 field trial applications for GM grasses. The vast majority of these have been for *Agrostis stolonifera* in the US and Canada. Indeed there is currently a petition pending in the US for the deregulation of glyphosate tolerant *A. stolonifera* from Monsanto and Scotts (Appendix VI).

3.4.3 Summary of containment issues relating to grasses

Gene flow from GM grasses is a concern because:

- out-breeding, wind borne pollen is capable of travelling long distances
- grasses are perennial
- cultivated species are undomesticated genotypes
 - will hybridise with wild species
 - will hybridise with close relatives, which may be weedy species
- some species have wind borne seed
- some species are capable of vegetative propagation

4. Review of Transgenic Methodologies for the ‘Bio-Pharming’ of Pharmaceutical Products

The adoption of plants as generators for industrial and pharmaceutical compounds is a rapidly expanding area of development. The production of plant based pharmaceuticals (“pharming”) offers many benefits compared to conventional mammalian cell culture techniques (Webster 2004). Plant based platforms are less costly with increased flexibility in terms of scale and storage and can return high yields (Table 4.1). The benefits are such that the World Health Organisation are now developing plant based production of antibodies for diseases such as rabies in collaboration with the biotech industry (WHO 2004), and the potential commercial market is estimated to grow at a compound annual growth rate of 104.3% over the period 2005-2011 (Figure 4.1) (Webster 2004).

Plant based pharmaceuticals present a different set of problems for the regulators (Howard & Donnelly 2004; Stewart & Knight 2005). The contamination of crops destined for consumption by humans or livestock by genes that express potentially

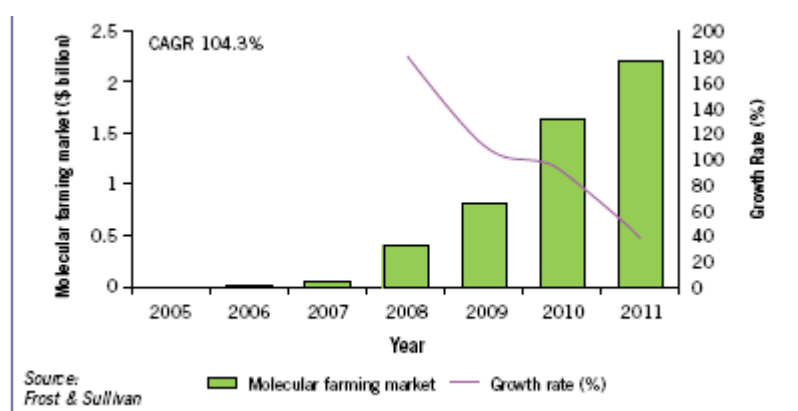


Figure 4.1 Forecast for the total North American market for plant biopharmed products for pharmaceutical use (Source: Webster 2004)

Table 4.1 Comparison of the features of recombinant protein production for the various bioreactor systems currently available (modified from Fischer *et al.* 1999b and Walker *et al.* 2005)^aIn terms of large-scale fermentors and associated equipment; ^bIn relation to contamination of human therapeutic proteins with viral sequences, oncogenes or endotoxins

Features	Production System					
	Bacteria	Yeast	Mammalian cell culture	Transgenic animals	Transgenic plants	Transgenic microalgae
Production time	Short	Medium	Long	Long	Long	Short
Production cost	Medium	Medium	High	High	Low	Very low
Scale up cost	High ^a	High ^a	High ^a	High ^a	Low	Very low
Storage cost	Low	Low	High	High	Low	Low
Storage method	-20°C	-20°C	Liquid N ₂	Liquid N ₂	Room temp	Room temp
Production scale	Limited	Limited	Limited	Limited	Worldwide	Worldwide
Propagation	Easy	Easy	Difficult	Feasible	Easy	Very easy
Distribution	Feasible	Feasible	Difficult	Difficult	Easy	Easy
Delivery vehicle	No	No	No	Yes	Possible	Possible
Gene size	Unknown	Unknown	Limited	Limited	Not limited	Not limited
Glycosylation	Absent	Incorrect	'Correct'	'Correct'	'Correct'	'Correct'
Multimeric protein assembly	No	No	No	Yes	Yes	Yes
Protein yield	Medium	High	Medium-high	High	High	Unknown
Risk ^b	Yes	Unknown	Yes	Yes	Unknown	Unknown
Safety	Low	Unknown	Medium	High	High	High
Ethical concerns	Low	Medium	Medium	High	Medium	Medium

harmful compounds is a cause for real concern, as has already been demonstrated by the 'Starlink' episode. A number of novel approaches are being investigated and adopted in order to reduce this risk. Physical containment strategies discussed in Section 3 of this report also apply to bio-pharming crops. In addition, various biological methods are available to reduce or eliminate gene transfer via pollen or any other route, with the choice of species being the most obvious consideration.

Bio-pharming systems have been developed for many existing crop species since there are established harvesting and processing structures in place and a high level of knowledge on the biology and genetic modification of many crop species. Following concerns regarding the extra levels of containment required for bio-pharma crops (Andow 2004), some groups have chosen to use non-crop plants as the basis of their bio-pharming technology as these will be easier to separate from existing agricultural and food processing systems and so will be less likely to enter the food chain.

4.1 Conventional crop species

Prior to the development of alternative species as production systems, bio-pharming technology was initially developed in conventional crops. The existence of previous knowledge and experience of the cultivation, harvesting and processing and of the genetics and breeding of conventional crops makes them attractive platforms for this technology (Stoger *et al.* 2002, 2005; Schillberg *et al.* 2005), providing containment issues can be addressed (Andow 2004). In addition, many conventional crops offer potential for the development of “edible vaccines” (Kirk *et al.* 2005) and medicines for both human (Gomord *et al.* 2005) and livestock use (Dus Santos & Wigdorovitz 2005; Streatfield 2005).

4.1.1 Pharmed products on the market

There are several bio-pharmed products currently on the market (Howard 2005; Howard & Hood 2005), most of which have been developed by Prodigene and are available through Prodigene and Sigma.

The first commercialised plant derived recombinant product was the glycoprotein avidin (Hood *et al.* 1997; Witcher *et al.* 1998; Zhong *et al.* 1999). This is a diagnostic reagent used in tests for human health and is usually sourced from the whites of chicken eggs. The gene coding the protein was introduced into maize and engineered to express the protein in the seed. The production system was calculated to be 100 times less expensive than chicken egg based production and the dried grain could be stored for years without any loss of activity (Howard 2005). Prodigene have a patent (US 5767379) relating to the commercial production of avidin in plants.

Prodigene also use maize as a production system for β -Glucuronidase (GUS). This is a visual marker used in transgenic plant research and is marketed by Sigma. The US patent 5804694 relates to Prodigene’s commercial production of this compound in plants

A more recent addition to the market (2004) is trypsin, a protease that has a wide range of uses from food processing, digestive aids and detergents, and is used as a research and tissue culture agent. As such, trypsin has a much larger market than avidin and β -Glucuronidase and trypsin represents the first large-scale production of a bio-pharmed reagent. It is usually derived from bovine or porcine pancreas cells and has been difficult to express artificially, as any protease by its nature degrades the proteins in its host cells (Howard 2005). Prodigene have developed a trypsin production system in maize, again expressing the product in the seed (Woodard *et al.* 2003) and have patented their protease plant production system (US 6087558). A plant-derived product has advantages in that there is no risk of contamination of viruses from animal cells and animal-free sources of many reagents are sought (Horn *et al.* 2004). This GM-derived product is commercially available as TrypZean via Sigma (catalogue number T3568/T3449) and directly via Prodigene.

The next new product developed by this company is aprotinin. This is a protease inhibitor that is normally derived from bovine lungs, and is used to reduce blood loss and the need for excess blood transfusion during open-heart surgery. The transgenic product is expressed in maize seed and has been proven to be both chemically and functionally identical to the bovine form (Howard 2005) (US 5824870). Issues with limitations on the supply of certified BSE free sources of aprotinin and the chemical similarity of Prodigene's product have facilitated the commercial development of this product. It is currently marketed as Trasylol by Bayer Pharmaceuticals and as AproliZean by Prodigene.

All of the Prodigene products are generated via their maize production platform where the transgene product is expressed in the seed. These crops are raised in the US under the regulatory control of the Animal and Plant Health Inspection Service (APHIS). Since 1997, Prodigene has been issued with over 70 permits from APHIS for field releases of maize expressing pharmaceutical and novel proteins. Details of the gene constructs of each of these releases are often not made publicly available due to business confidentiality. The containment strategies for transgenic maize crops in the US are relatively well established and APHIS requires that isolation distances around a modified maize crop must be at least one mile (1.6 Km). As the transgene product is expressed in the seed, the seed will be removed from the site

at harvest. Any remaining plant material is considered to contain insignificant amounts of the transgenic product and, as the derived proteins are not considered to have a toxic effect, is to be ploughed under. Maize is considered to be incapable of establishing itself as a weedy or feral plant as it cannot survive outside managed, arable conditions, thus seed escape is not considered an issue. In addition, there are no sexually compatible relatives of maize known to exist in the area where the trials are conducted at the Prodigene facility (NEPA document, http://www.aphis.usda.gov/brs/ph_permits.html).

Recent experimental studies on maize by Dow Chemical Company include the production of recombinant human IgA1 (hIgA1), and the first example of extensin-like Hyp/Pro conversion and O-linked arabinosylation for a recombinant therapeutic protein expressed in transgenic plants (Karnoup *et al.* 2005). Other recent studies using maize as a production system (Farinas *et al.* 2005a) includes the expression of recombinant human proinsulin in the endosperm (Farinas *et al.* 2005b).

Other companies with production strategies similar to Prodigene include Ventria Bioscience (<http://www.ventria.com>) who recently announced (19 July 2005) the introduction of a new plant-derived recombinant growth factor based on lactoferrin that improves productivity and safety. Their product, Lacromin™ is a strong growth factor and outperforms Transferrin, a common animal-derived growth factor. Lactoferrin is an iron-building human milk protein with anti-bacterial, anti-fungal, anti-viral, and anti-inflammatory properties. It occurs in low quantities in cows' milk and has also been expressed in Javanica rice (Rachmawati *et al.* 2005), in *Aspergillus* and in tobacco suspension cells (Horn *et al.* 2004).

Ventria's production platform is based on transgene expression in the seed of rice and barley crops and they have conducted US field trials of GM versions of these crops for the production of lactoferrin and other compounds. Most recently they report an improved method for the production of human lysozyme in rice (Hennegan *et al.* 2005). Meristem Therapeutics are also attempting to commercialise a lactoferrin product from maize but this product is still in clinical trials (Horn *et al.* 2004).

Ventria has produced five transgenic rice crops and one barley crop in the US. Expression of the transgene occurs in the seed of both. The containment strategies for transgenic rice crops are also well established in the US and APHIS requires an isolation distance of 0.25 miles (0.4 Km) from any other rice crop and a fallow zone of 50 feet (15 m) around the crop itself. Rice has a less than 1% out-crossing rate and there are no major rice crops grown in the vicinity of the Ventria facility. There are no wild rice species present in the US but a small weed problem exists with red rice. Ventria are required to monitor their site to ensure that no weedy rice is located within 0.25 miles (0.4 Km) of their crop. The crop is also inspected for infection with any insect or pathogenic pest. Harvesting, storage and processing to be carried out in dedicated equipment on site and the fields are to be burnt following harvest to degrade any plant material and remaining seed (not something now allowed in the UK). The cultivar selected for the trial is not competitive against weeds, has seed that lacks dormancy and loses viability quickly. In addition, the transgenic trait confers no selective advantage to the crop (NEPA document, http://www.aphis.usda.gov/brs/ph_permits.html).

Other recent experimental studies on rice include the production of the glucagon-like peptide that has potential in diabetes therapy (Sugita *et al.* 2005, Yasuda *et al.* 2005), a mammalian transglutaminase (Capell *et al.* 2004) and expression of cedar pollen induced T-cell epitopes (Takagi *et al.* 2005).

The containment strategies for GM barley are similar to those required for a transgenic rice crop. Barley is 99% self-pollinating and is rarely visited by insects. A fallow zone of 50 feet (15 m) is required around the crop and an isolation distance of 0.25 miles (0.4 Km) from the nearest barley crop. As the transgene is expressed in the seed, the remaining stubble is considered to contain insignificant amounts of the transgene product and may be ploughed under. The field must also be burnt when the conditions allow. Seed dormancy is not observed in barley and there are no sexually compatible wild relatives in the area of the Ventria facility. Dedicated handling equipment is again required and processing must take place on site (NEPA document, http://www.aphis.usda.gov/brs/ph_permits.html).

All plants expressing pharmaceutical products in the US are regulated and monitored by APHIS and as such an application for deliberate field trial must be made and the trial must be carried out in accordance with the specifications set out when the licence is issued. There are no plans to deregulate crops expressing pharmaceutical products in the US (Ma *et al.* 2005a).

4.1.2 Pharmed products close to the market

There are numerous bio-pharmed products that are currently close to the market. These include the antibody CaroRx, collagen from tobacco, human gastric lipase, and vaccines induced in edible crops (Horn *et al.* 2004). A summary of the status of some of these programmes is provided below (Table 4.2) (Ma *et al.* 2005a).

It should be noted that the early emphasis on the idea of using fruit or vegetables directly as 'edible vaccines' has now been superseded by realisation of the difficulty of controlling the dosage and the problems associated with separating such GM products from the non-GM food chain. The present terminology is 'orally active thermostable vaccines' and the future programmes in this area are likely to involve a processed rather than a fresh product.

Table 4.2 Plant-derived pharmaceutical proteins that are closest to commercialization for the treatment of human diseases (From Ma *et al.* 2005a)

Crop	Class	Product	Disease	Company/Organisation	Status
Transgenic <i>Arabidopsis</i>	Dietary	Human intrinsic factor	Vitamin B12 deficiency	Cobento Biotech AS	Phase I
Transgenic lettuce	Vaccine	Hepatitis B virus surface antigen	Hepatitis B	Thomas Jefferson University/Polish Academy of Sciences	Phase I
Transgenic maize	Dietary	Lactoferrin	Gastrointestinal infections	Meristem Therapeutics	Phase I
Transgenic maize	Therapeutic enzyme	Gastric lipase	Cystic fibrosis, Pancreatitis	Meristem Therapeutics	Phase II
Transgenic potato	Vaccine	<i>E. coli</i> heat labile toxin	Diarrhoea	Tacket <i>et al.</i> 1998	Phase II
Transgenic potato	Vaccine	Hepatitis B virus surface antigen	Hepatitis B	Richter <i>et al.</i> 2000	Phase I
Transgenic potato	Vaccine	Norwalk virus capsid protein	Norwalk virus infection	Tacket <i>et al.</i> 2000	Phase I
Transgenic tobacco	Antibody	CaroRx	Dental caries	Planet Biotechnology Inc	Phase II
Transgenic tobacco	Vaccine	<i>E. coli</i> heat labile toxin	Diarrhoea	Prodigene Inc	Phase I
Viral vectors in <i>Nicotiana benthamiana</i>	Antibody	Various single chain Fv antibody fragments	Non-Hodgkin's lymphoma	Large Scale Biology Corp	Phase I
Viral vectors in spinach	Vaccine	Rabies glycoprotein	Rabies	Yusibov <i>et al.</i> 2002	Phase I

4.1.3 Tobacco

Due to the long history of genetic modification in tobacco, this was the most obvious first target species for 'pharming' application and indeed, more transgenes have been expressed in tobacco than all other species combined (Grevich & Daniell 2005). Tobacco is a very efficient production system, with one plant producing a million seeds and one acre producing in excess of 40 metric tonnes of leaves per year (Cramer *et al.* 1999). As a bioreactor species, tobacco is significantly less costly than standard *E. coli* production systems (Kusnadi *et al.* 1997) and as it is neither a food nor a feed crop it is unlikely to contaminate the food chain (Grevich & Daniell 2005). In addition, there is an established process for harvesting leaf material and this can be done before flowering takes place. Several companies are using tobacco for pharmaceutical production.

One example of this is the recent announcement (12 July 2005) by Large Scale Biology Corporation (LSBC) and Planet Biotechnology of increased production of Planet's lead product, CaroRx™, a plant-made antibody to control dental caries. CaroRx™ is the world's first recombinant plant-made antibody (plantibody) and has been shown in clinical studies to prevent the adhesion to the tooth surface of the decay causing bacteria *Streptococcus mutans* (Figure 4.2).

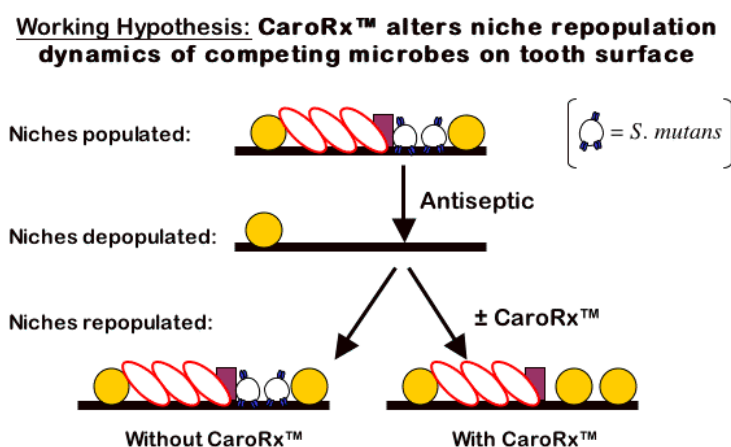


Figure 4.2 Working hypothesis for mode of action of CaroRx™
(Source: Planet Biotechnology)

Many species of bacteria inhabit the mouth and teeth (Figure 4.2) but most of these are harmless. Antiseptic treatment will kill and remove most of these bacteria including *S. mutans* from between the teeth. The removal of bacterial leaves an 'ecological niche' previously occupied by *S. mutans* and this will be recolonised under normal circumstances. With CaroRx™ treatment recolonisation of *S. mutans* is blocked by preventing the adhesion of the bacteria to the surface of the teeth. Colonization of other oral bacteria occurs unimpeded and after a sufficient period the niche once occupied by *S. mutans* is occupied by some other organism, or is altered in some other way to exclude *S. mutans*.

Planet's tobacco plants expressing the proprietary CaroRx™ protected SIgA™ are being grown in Kentucky and extracted by Large Scale Biology Corporation (LSBC) (www.lsbcb.com) at its Owensboro manufacturing facility in Kentucky. It was stated in a press release on 12th July 2005 (http://www.lsbcb.com/pdfs/Planet_LSBC_7_12_2005.pdf) that 'CaroRx™ is currently approved for sale as a medical device in the European Union', although no further details of this approval are known and this statement is no longer (13th December 2005) on their web site. LSBC is a biotechnology company developing and bio-manufacturing bio-therapeutics, vaccines and industrial proteins for important life science industry markets. They have an extensive patent portfolio in areas relevant to this technology (Table 4.3), and have been financially aided in 2005 by the commercial arm of the University of Kentucky. LSBC use transiently expressed viral vectors to induce recombinant protein production in non-transformed *Nicotiana* (see section 4.2).

Planet Biotechnology Inc. (www.planetbiotechnology.com) is a privately held, clinical-stage company whose mission is 'to discover, develop and commercialise new monoclonal-antibody-based therapeutic and preventative products to meet significantly underserved medical needs'.

Icon Genetics (<http://www.icongenetics.com/>) are also using tobacco in a similar project announced in August 2005. In a joint experiment in collaboration with the Kentucky Tobacco Research and Development Center (KTRDC), University of

Table 4.3 The Large Scale Biology Corporation patent portfolio

Title	US Patent	Issue Date	Technology
Plant Viral Vectors Having Heterologous Subgenomic Promoters for Systemic Expression of Foreign Genes	5316931	31 May 1994	GENEWARE®, Plant
Recombinant Plant Viral Nucleic Acids	5589367	31 December 1996	GENEWARE®, Plant
Viral Amplification of Recombinant Messenger RNA in Transgenic Plants	5811653	22 September 1998	GENEWARE®, Plant
Recombinant Plant Viral Nucleic Acids	5866785	2 February 1999	GENEWARE®, Plant
Recombinant Plant Viral Nucleic Acids	5889190	30 March 1999	GENEWARE®, Plant
Viral Amplification of Recombinant Messenger RNA in Transgenic Plants	5889191	30 March 1999	GENEWARE®, Plant
The Cytoplasmic Inhibition of Gene Expression by Viral RNA	5922602	13 July 1999	GENEWARE®, Plant
Viral Amplification of Recombinant Messenger RNA in Transgenic Plants	5965794	21 October 1999	GENEWARE®, Plant
Production of Peptides in Plants as Viral Coat Protein Fusions	5977438	2 November 1999	GENEWARE®, Plant
Viral Amplification of Recombinant Messenger RNA in Transgenic Plants	6462255	8 October 2002	GENEWARE®, Plant
The Cytoplasmic Inhibition of Gene Expression by Viral RNA	6479291	12 November 2002	GENEWARE®, Plant
Viral Expression Vectors	6656726	2 December 2003	GENEWARE®, Plant
Production of Peptides in Plants as Viral Coat Protein Fusions	6660500	9 December 2003	GENEWARE®, Plant
Cytoplasmic Inhibition of Gene Expression in Transfected Plants by Tobravirus Vector	6700040	2 March 2004	GENEWARE®, Plant
Cytoplasmic Inhibition of Gene Expression and Expression of a Foreign Protein in a Monocot Plant by a Plant Viral Vector	6800748	5 October 2004	GENEWARE®, Plant
Production of a Parvovirus Vaccine in Plants as Viral Coat Protein Fusions	6730306	4 May 2004	Vaccines
Method for Conferring Herbicide, Pest or Disease Resistance in Plant Hosts	6303848	16 October 2001	Genomics
Method of Compiling a Functional Gene Profile in a Plant by Transfecting a Nucleic Acid Sequence of a Donor Plant into a Different Host Plant in an Anti-Sense Orientation	6426185	30 July 2002	Genomics
Process for Isolating and Purifying Viruses, Soluble Proteins and Peptides From Plant Sources	6033895	7 March 2000	Biomanufacturing
Process for Isolating and Purifying Viruses, Soluble Proteins and Peptides From Plant Sources	6037456	14 March 2000	Biomanufacturing
Method for Recovering Proteins From the Interstitial Fluid of Plant Tissues	6284875	4 September 2001	Biomanufacturing
Method for Recovering Proteins from the Interstitial Fluid of Plant Tissues	6441147	27 August 2002	Biomanufacturing
Method for Recovering Proteins from the Interstitial Fluid of Plant Tissues	6617435	9 September 2003	Biomanufacturing

Kentucky, Lexington, Icon will field trial transgenic plants with genetically engineered chloroplasts containing a phenylalanine ammonia lyase (PAL) gene from *Arabidopsis thaliana*. A release permit has been obtained from APHIS, the United States Department of Agriculture's Animal and Plant Health Inspection Service. According to Zaitlin *et al.* (2004) KTRDC believe that an F₁ hybrid strategy can prevent transgene escape. For production of industrial or pharmaceutical proteins, the maternal (tobacco) cultivar is transformed and homozygous transformants with high-level expression are selected and crossed with other *Nicotiana* species. It is the resulting sterile, morphologically distinct, F₁ hybrids that are grown in the field (Figure 4.3).



Figure 4.3 *Nicotiana* crop-producing materials for medical and other applications

The crop is close-grown and machine-harvested, with up to five harvests per season. Tobacco plants are harvested at night to ensure lowest possible temperatures of the plants for transfer to the bioprocessing facility. (Source: <http://www.uky.edu/KTRDC/TOB%20Tech9%2005.pdf>).

Overexpression of the PAL gene in tobacco chloroplasts is aimed at both pharmaceutical and industrial applications. The purified enzyme can serve as a drug for the treatment of the inherited disease phenylketonuria (PKU) and also serves as a biocatalyst for industrial biochemical synthesis. In addition, strong expression of PAL leads to accumulation of metabolites from the phenylpropane pathway, which

Table 4.4 US field trials of GM tobacco expressing pharmaceutical products

CBI: confidential business information. (Source: APHIS <http://www.aphis.usda.gov/brs/pharmaceutical.html>)

Permit	Date Issued	Company	Modification
99-041-02r	8 March 1999	CropTech	CBI
99-041-01r	8 March 1999	CropTech	CBI
00-070-01r	19 April 2000	CropTech	CBI from Man
00-070-02r	19 April 2000	CropTech	CBI from Man
01-074-01r	11 May 2001	CropTech	CBI
01-074-02r	11 May 2001	CropTech	CBI
02-080-01r	7 May 2002	CropTech	CBI from Man
03-063-01r	15 May 2003	Chlorogen Inc	Serum albumin from Man
04-044-01r	28 April 2004	Planet Biotechnology	Antibody from Mouse Antibody from <i>Oryctolagus cuniculus</i>
04-114-04r	25 May 2004	Chlorogen Inc	Albumin from Man; Insulin-like growth factor from Man; Interferon from Man; Mullerian inhibiting substance from Man
04-355-01r	19 May 2005	Chlorogen Inc	CBI from Man; CBI from Cow
05-061-01r	7 June 2005	University of Kentucky	Phenylalanine ammonia lyase from <i>Arabidopsis thaliana</i>
05-053-01r	8 June 2005	Planet Biotechnology	Common cold antibody from CBI; Tooth decay antibody from Mouse; Tooth decay antibody from <i>Oryctolagus cuniculus</i>
05-087-01r	7 July 2005	Planet Biotechnology	Tooth decay antibody from Mouse

are also of commercial value. It is claimed that chloroplast-located transgenes generally do not spread into the environment via pollen flow. Moreover, the genetically engineered plants do not contain antibiotic resistance genes, since they were created using ICON's proprietary resistance marker removal technology.

A modified version of the tobacco production system is that reported by Menassa *et al.* (2001) who describe the use of a hybrid male-sterile low-alkaloid tobacco (MSLA) for the production of human interleukin-10.

The majority of these products have been produced in field trials in the US. Since 1992 there have been 16 field trial applications for tobacco expressing pharmaceutical and industrial proteins in the US. Fourteen of these have been granted to CropTech, Planet Biotechnology, Chlorogen Inc, and the University of Kentucky (Table 4.4). Further details of these field trials are listed in Appendix IV.

Table 4.5 European field trials of tobacco expressing pharmaceutical products

(Source: http://gmoinfo.jrc.it/gmp Browse_geninf.asp)

Permit	Country	Company	Modification
B/FR/98/05/07	France	Seita – Institut du Tabac	Synthesis of antibodies
B/FR/97/05/17	France	Biocem SA – Limagrain Group	Synthesis of collagen
B/FR/99/02/13	France	Meristem Therapeutics	Synthesis of collagen
B.FR/95/02/18-CON	France	Biocem SA – Limagrain Group	Synthesis of dog gastric lipase
B/FR/96/01/10-CON	France	Biocem SA – Limagrain Group	Synthesis of dog gastric lipase
B/FR/96/01/11-CON	France	Biocem SA – Limagrain Group	Synthesis of dog gastric lipase
B/FR/97/05/15	France	Biocem SA – Limagrain Group	Synthesis of dog gastric lipase
B/FR/97/05/16	France	Biocem SA – Limagrain Group	Synthesis of dog gastric lipase
B/FR/95/02/17-CON	France	Biocem SA – Limagrain Group	Synthesis of human alpha-1 antitrypsin
B/FR/96/01/12-CON	France	Biocem SA – Limagrain Group	Synthesis of rabies virus G glycoprotein
B/ES/97/32	Spain	Biocem SA, Clause Iberica	Synthesis of collagen
B/ES/97/33	Spain	Biocem SA, Clause Iberica	Synthesis of collagen
B/ES/97/24	Spain	Tézier Ibérica sl – Limagrain Group	Synthesis of dog gastric lipase
B/IT/01/02	Italy	Plantechno Srl, Università Cattolica S Cuore, Facoltà di Agraria, Istituto di Botanica e Genetica Vegetale	Synthesis of human glucocerebrosidase protein Synthesis of pharmaceutical compounds

APHIS demands that field trials of transgenic tobacco expressing pharmaceutical products are topped to prevent flowering. Non-transgenic crops less than one mile from the field site are also prevented from flowering.

There have also been a number of trials in Europe of transgenic tobacco expressing pharmaceutical products (Table 4.5) all of which took place between 1995 and 2001 and no trials have been conducted more recently. The containment strategies in place during these trials are no longer available through the European Commission's Joint Research Centre website as the trials are not recent.

There are numerous other studies on transgenic tobacco (Elliott *et al.* 1996; Alli *et al.* 2002) expressing a wide range of pharmaceutical products. These are not yet at a commercial stage of development and summarised below:

Anthrax antigen: Watson *et al.* 2004; Aziz *et al.* 2002

Antibodies: Almquist *et al.* 2004; Bakker *et al.* 2001; Bruyns *et al.* 1996; Cabanes-Macheteau *et al.* 1999; Frigerio *et al.* 2000; Galeffi *et al.* 2005; Kathuria *et al.* 2002; Ko *et al.* 2005; Le Gall *et al.* 1998; Magee *et al.* 2004; Makvandi-Nejad *et al.* 2005; Nuttall *et al.* 2002; Rodriguez *et al.* 2005; Schillberg *et al.* 1999; Stevens *et al.* 2000; Vaquero *et al.* 2002; Weintraub *et al.* 2005; Wu *et al.* 1997

Antigens: Daniell *et al.* 2001; Ehsani *et al.* 2003; Elbers *et al.* 2001; Ramirez *et al.* 2003; Rukavtsova *et al.* 2003.

Birch pollen allergen Bet v 1: Krebitz *et al.* 2000

Bovine growth hormone (bGH): Oh *et al.* 2003

Cholera toxin B subunit: Wang *et al.* 2001;

Colorectal cancer antigen GA733-2: Verch *et al.* 2004

Desmodus rotundus salivary plasminogen activator alpha1: Schiermeyer *et al.* 2005

Escherichia coli enteropathogenic pilin subunit A: da Silva *et al.* 2002

Escherichia coli heat-labile enterotoxin (LTB), non-toxic B subunit: Kang *et al.* 2003

Hantavirus serotype Puumala, Nucleocapsid protein: Khattak *et al.* 2004

Helicobacter pylori strain ZJC02, UreB antigen: Gu *et al.* 2005

Honeybee royal jelly MRJP1: Judova *et al.* 2004

Human alpha-lactalbumin: Takase *et al.* 1998

Human acetylcholine esterase-R: Geyer *et al.* 2005

Human CD 14: Blais & Altosaar 2005

Human cytomegalovirus glycoprotein B: Tackaberry *et al.* 1999, 2003; Wright *et al.* 2001

Human epidermal growth factor: Wirth *et al.* 2004

Human erythropoietin: Cheon *et al.* 2004

Human granulocyte-macrophage colony stimulating factor: Sardana *et al.* 2002

Human growth hormone: Leite *et al.* 2000

Human hepatitis B virus core antigen: Tsuda *et al.* 1998; Valdes *et al.* 2003; Yano & Takeoshi 2004

Human interferon: Leelavathi & Reddy 2003

Human immunodeficiency virus, diagnostic reagent: Schunmann *et al.* 2002

Human immunodeficiency virus type I (HIV-1) p24 capsid protein: Zhang *et al.* 2002; Obregon *et al.* 2006

Human insulin-like growth factor precursor prohormone IGF-1B: Panahi *et al.* 2003, 2004

Human islet autoantigen glutamic acid decarboxylase: Porceddu *et al.* 1999; Avesani *et al.* 2003

Human lactoferrin: Salmon *et al.* 1998; Samyn-Petit *et al.* 2003

Human papillomavirus type 16 virus-like proteins: Biemelt *et al.* 2003; Liu *et al.* 2005, Varsani *et al.* 2003

Human placental β -glucosidase: Reggi *et al.* 2005

Human somatotropin: Staub *et al.* 2000

Hydroxylated human homotrimeric collagen I: Ruggiero *et al.* 2000; Merle *et al.* 2002; Osorio 2004

Immunogenic Puumala virus nucleocapsid protein: Kehm *et al.* 2001

Interleukin-18: Zhang *et al.* 2003

Interleukin-4: Ma *et al.* 2005b

K88 fimbriae, major subunit: Huang *et al.* 2003b; Joensuu *et al.* 2004

Measles virus, haemagglutinin protein: Huang *et al.* 2001, Webster *et al.* 2005

***Plasmodium falciparum* C-terminal region of merozoite surface protein (PfMSP1(19)), a potential malaria vaccine candidate:** Ghosh *et al.* 2002

Preprorizin: Sehnke & Ferl 1999

Porcine epidemic diarrhoea virus, protein: Bae *et al.* 2003; Kang *et al.* 2004, 2005b

Porcine parvovirus, Capsid protein VP2: Rymerson *et al.* 2003

Porcine relaxin: Buswell *et al.* 2005

Rabies virus, surface glycoprotein: Ashraf *et al.* 2005

Recombinant dog gastric lipase: Gruber *et al.* 2001

Rinderpest virus, haemagglutinin protein: Khandelwal *et al.* 2003

Rotavirus VP6: Birch-Machin *et al.* 2004

SARS-coronavirus (CoV) spike protein: Pogrebnyak *et al.* 2005

Single-chain human interleukin-12: Gutierrez-Ortega *et al.* 2004

***Staphylococcus aureus*, staphylokinase:** Dobrowolska *et al.* 2004

***Toxoplasma gondii*, surface antigen:** Clemente *et al.* 2005

Tetanus toxin Fragment C: Tregoning *et al.* 2003, 2005

Thrombomodulin: Schinkel *et al.* 2005

Xylanase: Leelavathi *et al.* 2003

4.1.4 Alfalfa

Alfalfa (or lucerne) is a perennial plant that does not die after harvesting but grows back to maturity in five weeks, without flowering, sexual crossing or producing seeds. The new leaves generated after harvesting are identical, perfectly natural clones of the ones they replace, thus alfalfa is a highly efficient protein production system. In addition, vegetative propagation allows for ultra-fast multiplication by stem-cutting, producing identical clones. Alfalfa can be harvested at least ten times a year for ten years or more, providing 10g of recombinant protein for every square metre of greenhouse space.

The most advanced production system using this crop is that developed by Medicago in Canada (<http://www2.medicago.com/en/>). Alfalfa plants are grown in hi-tech greenhouses under their proprietary system named Proficia™. This system is claimed to offer high volume potential of plant-based technology together with the safety and control of confined environment production.

Medicago has currently four products under early stage development. Preclinical trials are planned to start in 2005 for two products: monoclonal antibodies and plasmatic proteins. A recently announced collaboration (2 June 2005) with Intervexion Therapeutics (<http://www.intervexion.com/>) will use Medicago's technology for the production of monoclonal antibodies therapeutics designed to treat drug abuse. Intervexion has received a \$3 million grant to conduct clinical trials for the first antibody treatment for addiction to the drug known as phencyclidine, or PCP. It is claimed that that working closely with FDA and NIDA will allow an accelerated clinical program to get the drug approved in three to four years.

Earlier this year (31 January 2005) Medicago announced a research collaboration with Bayer CropScience to assess the feasibility of using the Proficia™ system for the production of an undisclosed human therapeutic protein.

Table 4.6 US field trials of GM alfalfa expressing pharmaceutical products(Source: APHIS <http://www.aphis.usda.gov/brs/pharmaceutical.html>)

Permit	Date Issued	Company	Modification
94-362-02r	12 April 1995	University of Wisconsin	Amylase from <i>Bacillus licheniformis</i> ; Lignin peroxidase from <i>Phanerochaete chrysosporium</i>
97-052-02r	23 April 1997	University of Wisconsin/Madison	Cellulase; Inositol hexaphosphate phosphohydrolyase from <i>Aspergillus niger</i> ; Lignin peroxidase
93-088-02r	4 June 1993	University of Wisconsin	Alpha-amylase from <i>Bacillus licheniformis</i> ; Lignin peroxidase from <i>Phanerochaete chrysosporium</i>
92-183-01r	10 October 1992	Noble Foundation	Cholera toxin B from <i>Vibrio cholera</i>

Since 1992 there have been four field trials of transgenic alfalfa in the US carried out by the University of Wisconsin and the Noble Foundation (Table 4.6). Further details of these trials are listed in Appendix IV.

The Medicago crop of alfalfa was grown under physical containment. For field release of GM alfalfa APHIS requires an isolation distance of 900 feet to the nearest alfalfa crop. Most commercial crops of alfalfa are harvested every 20-40 days to prevent seed formation.

To date there have been no field trials in Europe of alfalfa expressing pharmaceutical proteins.

Other projects using a transgenic alfalfa system (D'Aoust *et al.* 2004a, 2004b; Vézina *et al.* 2002) include:

Antibodies: Bardor *et al.* 2003; Busse *et al.* 2002; Khoudi *et al.* 1999

Bovine rotavirus, VP4 protein: Wigdorovitz *et al.* 2004

Classical Swine Fever Virus, E2 glycoprotein: Legocki *et al.* 2005

Foot and mouth disease virus, structural protein VP1: Wigdorovitz *et al.* 1999

Foot and mouth disease virus, immunogenic epitope: Dus Santos *et al.* 2002; Dus Santos & Wigdorovitz 2005

Human group A rotavirus, VP6 protein: Dong *et al.* 2005

Mannheimia haemolytica, leukotoxin gene from: Ziauddin *et al.* 2004

Root exudation of recombinant protein: Tesfaye *et al.* 2005

Promising results from *M. truncatula* have also been reported (Abranches *et al.* 2005).

4.1.5 Sugarcane

Another species adopted for use in bio-pharming techniques is sugar cane as it is a non-flowering crop. Wang *et al.* (2005b) developed 200 independent sugarcane lines producing the human cytokine granulocyte macrophage colony-stimulating factor (GM-CSF). Analysis of recombinant protein accumulation and activity levels of GM-CSF protein ranged from undetectable to 0.02% of total soluble protein. Human bone marrow cells (TF-1), which require GM-CSF for cell division, proliferated when growth media was supplemented with transgenic sugarcane extracts. Comparison to purified commercially produced GM-CSF indicated the sugarcane-produced protein had essentially identical activity levels. The field trial was conducted in Hawaii and the trial plants did not flower, thus no pollen or seed was produced from the crop. In addition, the test plants were dried, burnt and buried to prevent the transgenic sugarcane entering the environment or food supply. It was suggested therefore that sugarcane might provide a highly secure system for biofactory production of pharmaceutical proteins. As a food crop, regulations need to be in place to ensure the containment of transgenes from pharma-sugarcane.

In a related publication (<http://tlo.tamu.edu/tlo/documents/INDR/1436.pdf>) Texas A&M University are offering to produce any recombinant protein in sugar cane. They claim that sugarcane is an ideal bio-pharming crop for the following reasons:

- Sugarcane displays the highest biomass per acre of any annual crop.
- Plant materials are not part of the food chain since they are seldom consumed directly by humans, unlike corn or rice.
- Sugarcane economics can be leveraged by a variety of valuable by-products, e.g., the biogases following crushing can be burned to generate power and the sucrose can be used for the production of ethanol.
- Does not cross-pollinate since it can be grown in geographic areas where the plant does not flower.
- Protein expression demands relatively little post-transcriptional modification.
- Due to sugarcane's high-water content, protein purification is relatively simple.

- Plants harbour no animal pathogens.

Texas A&M University System have already (2003) signed a license agreement with proCANE LLC, a subsidiary of ECOR Corp. of Sedona, Arizona, to produce pharmaceutical-grade proteins in sugarcane plants.

Since 1992 there has been only one field trial of transgenic sugarcane expressing pharmaceutical products in the US. This was the Hawaii Agricultural Research Centre permit 01-306-01r issued on 11 January 2002. There have been no field trials in Europe.

4.1.6 Lettuce

Lettuce has also been adopted for pharmaceutical production, as it is another leafy species for which harvesting can occur prior to flowering. The following products have been isolated from transgenic lettuce:

Antibodies: Negrouk *et al.* 2005

Human growth factor: Zuo *et al.* 2001

Vaccine: Kapusta *et al.* 1999, 2001; Kawashima *et al.* 2001; Koprowski 2002; Legocki *et al.* 2005

Lettuce has advantages in that it is self-pollinating and can be commercially produced in large quantity under well-defined conditions with controlled hydroponic growth in animal pathogen free green houses (Figure 4.4). In terms of commercial development, Altor acquired intellectual property rights to the transgenic plant expression technology from Sunol Molecular Corporation (Figure 4.5) in 2005 and has a collaborative agreement with Dow Chemical Company on therapeutic antibody production in transgenic plants (<http://www.sunolmolecular.com/>).

As transgenic lettuce expressing pharmaceutical products can be grown in physical containment there have been no applications for field releases of pharma-lettuce in the US or in Europe.



Figure 4.4 Transgenic lettuce
(Source: www.sunolmolecular.com)

Recent relevant progress with this crop includes the first report of plastid transformation (Lelivelt *et al.* 2005), and high-level transient expression using an *A. tumefaciens* method (Joh *et al.* 2005; Negrouk *et al.* 2005) (see Section 4.2).

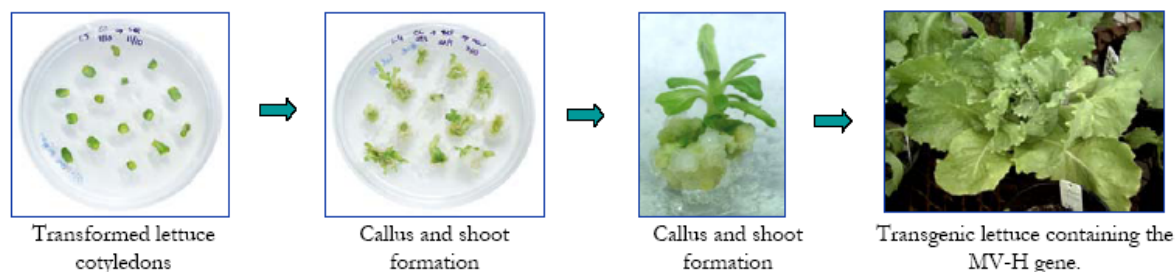


Figure 4.5 Lettuce transformation
(<http://www.burnet.edu.au/freestyler/gui/files/plantmvposter.pdf>)

4.1.7 Potato

Potato was one of the first species used for pharmaceutical production. In 1998 Axis Genetics, a private UK company, commissioned American Ag-Tec International to grow potatoes containing hepatitis B vaccine for clinical trials. Since then potato has been used to produce a wide range of pharmaceutical products:

Antibody: Artsaenko *et al.* 1998; Conrad U and Fiedler U 1998; De Wilde *et al.* 2002; Ehsani *et al.* 1997; Hendy *et al.* 1999

Antigen: Arakawa *et al.* 1999; Dogan *et al.* 2000; Joung *et al.* 2004; Kong *et al.* 2001; Lauterslager *et al.* 2001; Ma & Jevnikar 1999; Maloney *et al.* 2005; Shulga *et al.* 2004; Thanavala *et al.* 2005; Yu & Langridge 2001

Avian coronavirus infectious bronchitis virus: Wu *et al.* 2003b; Zhou *et al.* 2003, 2004

Bovine group A rotavirus, VP6 protein: Matsumura *et al.* 2002; Wu *et al.* 2003a

Cholera toxin: Choi *et al.* 2005

***Escherichia coli* enterotoxin (recLT-B), non-toxic B subunit:** Lauterslager *et al.* 2001

Foot and mouth disease virus structural protein VP1: Carrillo *et al.* 2001

Hepatitis B surface antigen: Thanavala *et al.* 2005

HIV diagnostic reagent: Schunmann *et al.* 2002

HIV-1 Tat transduction domain rotavirus enterotoxin fusion protein: Kim & Langridge 2004

Human interferon: Ohya *et al.* 2001, 2005

Human interleukin-2: Park & Cheong 2002

Human interleukin-4: Ma *et al.* 2005b

Human papillomavirus particles: Biemelt *et al.* 2003; Warzecha *et al.* 2003

Human serum albumin: Farran *et al.* 2002

Human β -amyloid peptide: Kim *et al.* 2003

Norwalk virus capsid protein: Tacket *et al.* 2000

Porcine epidemic diarrhoea virus, neutralizing epitope: Kim *et al.* 2005

Puumala virus nucleocapsid protein: Kehm *et al.* 2001

Rabbit hemorrhagic disease virus, structural protein VP60: Castanon *et al.* 1999, 2002

Sea anemone equistatin: Outchkourov *et al.* 2003

Swine-transmissible gastroenteritis coronavirus, spike protein: Gomez *et al.* 2000

The most commercially advanced of these projects are probably those of the two companies Quantum Tubers Corporation of Wisconsin (www.quantumtubers.com) and Planton (Kiel, Germany). This latter company specializes in the production of human anti-microbial peptides and claims an extraction rate of approximately 10 - 15mg of these compounds from one Kg of transgenic potato tubers (Figure 4.6). It is stated 'The proof of concept for the drug has been established, and pre-clinical trials with the product are ongoing. Meanwhile Planton has succeeded in establishing a co-operation with a big industrial partner to market its products.'



Figure 4.6 Potato plants *in vitro*

(Source: <http://www.biotech-online.com/artimg/a20051934236820.PDF>)

No evidence can be found of field trials of GM potatoes expressing pharmaceutical proteins. It must therefore be assumed that potatoes generated to express recombinant products are being produced in physical containment in greenhouses or controlled environment growth rooms.

There have been two field trials of transgenic potatoes in Germany expressing industrial compounds. These were both conducted by the Institute of Plant Genetics and Crop Plant Research. The first trial took place between 2002-4 of potatoes expressing non-plant carbohydrates but no further details of this trial can be found at present (notification number B/DE/02/138; <http://biotech.jrc.it/deliberate/DE.asp>). The second trial took place between 2003-4 of potatoes expressing non-plant spider silk proteins (notification number B/DE/02/146; <http://biotech.jrc.it/deliberate/DE.asp>). According to the notification report the risk of transgene escape was considered to be nil as no sexually compatible wild species are present in Europe and no other potato crop was raised near the release site (http://gmoinfo.jrc.it/gmp_browse_geninf.asp).

4.1.8 Other species

Other crop species used for pharmaceutical production include:

Arabidopsis: Downing *et al.* 2006; Gil *et al.* 2006; Wu *et al.* 2004a,b

Banana: Kumar *et al.* 2005

Carrot: Alli *et al.* 2002, Bouche *et al.* 2003, 2005; Marquet-Blouin *et al.* 2003; Porceddu *et al.*

1999; Sciutto *et al.* 2002

Lotus corniculatus: Kato *et al.* 2005

Lupin: Kapusta *et al.* 1999, Smart *et al.* 2003

Papaya: Sciutto *et al.* 2002

Pea: Perrin *et al.* 2000; Saalbach *et al.* 2001

Pigeon pea: Satyavathi *et al.* 2003

Spinach: Karasev *et al.* 2005, Sussman, 2003; Yusibov *et al.* 2002

Sunflower: Sciutto *et al.* 2002

Sweet potato: Berberich *et al.* 2005

Tomato: Fletcher *et al.* 2004; Huang *et al.* 2005; Jani *et al.* 2002; Kwon *et al.* 2003a; Ma *et al.* 2003; McGarvey *et al.* 1995; Mor *et al.* 2001; Pogrebnyak *et al.* 2005; Ruf *et al.* 2001; Sandhu *et al.* 2000; Shchelkunov *et al.* 2004

White clover: Lee *et al.* 2001, 2003

Of these plant species, there are only two that have been taken forward into field trials. There has been one field trial of tomato expressing a classified pharmaceutical protein in the US. This trial was carried out by Prodigene and the release permit was issued in 1998. In the Netherlands, Van der Have carried out a field trial of sunflower in 1995 expressing a variety of traits including albumin. This sunflower line was also modified to have male sterility.

The crop most recently suggested as a production system is flax. The newly established company Agragen announced plans in 2005 to develop transgenic plants expressing therapeutic proteins to be grown in North Dakota. Selection of flax as a production system is considered by Agragen to be ideal as it is self-pollinating and pollen travel a very short distance. In addition, flax pollen is short lived, reducing the risk of pollen mediated transgene escape. As flax is currently cultivated in North Dakota, a skills base exists in relation to farming and processing, however there are local concerns regarding the biological safety of the conventional flax crop.

The remainder of these expression systems are in the developmental stage at present.

4.1.9 Identity preservation

The use of food crops as production systems for recombinant proteins has been quite extensive, and although alternative crop species are undergoing continuous development, there still remains considerable focus on the conventional species. For this reason there are several suggestions about ways that phenotypic markers may be introduced into the crop to enable instant recognition of conventional crops expressing recombinant proteins and the use of inducible promoters to control expression of the product (Ma *et al.* 2005a). For example, alteration of fruit colour or fluorescent marker proteins such as the green fluorescent protein or DsRed could be added into the transgene cassette and be used not only to generate a visually distinct pharma crop, but also as a means of monitoring transgene escape without the need for molecular lab work (Ma *et al.* 2005a).

4.1.10 Summary of conventional crop species production platforms

Several products generated in GM plants are commercially available:

- products generated in maize (Prodigene)
 - Avidin
 - β -glucuronidase
 - Trypsin
- products generated in rice (Ventria)
 - lactoferrin

Numerous products are in clinical trials and are close to market release:

- products generated in tobacco
 - collagen
 - antibodies against tooth decay
 - antibodies against Non-Hodgkin's lymphoma (transient expression)
- products generated in maize
 - human gastric lipase
 - therapeutic enzymes
 - dietary supplements
- products generated in other crop species
 - vaccines against Hepatitis B and Norwalk virus in potato
 - vaccines against rabies in spinach (transient expression)
 - dietary supplements in *Arabidopsis*

Tobacco is widely used as a production platform. It is an efficient production system with an established knowledge base in genetics, manipulation, cultivation and production. It has been adopted as a production platform by Planet Biotechnology Inc, The Large Scale Biology Corporation, Icon Genetics and Chlorogen, Inc. Field trials have been conducted in the US and Europe.

Alfalfa and lettuce have also been adopted as production platforms. The leaves can be harvested repeatedly and flowering can be controlled. There

have been few field releases as both can easily be grown in physical containment.

A wide range of products have been expressed and produced in potato tubers. These are also mostly grown in physical containment.

Several other crops have also been adopted as production platforms but remain mostly experimental:

- *Arabidopsis*; banana; carrot; *Lotus*; lupin; papaya; pea; pigeon pea; spinach; sugarcane; sunflower; sweet potato; tomato; white clover

Development of morphological markers to distinguish pharma-expressing crops is suggested to aid identification and traceability without molecular techniques.

4.2 Transient expression

Expression of recombinant proteins in plants can be achieved by the development of stable, transgenic plant lines (as discussed above), or via the transient expression of a gene inserted into a non-GM plant by a vector. Transient gene expression enables the production of GM products from non-GM plants as it is based on the expression of traits in specific tissues (e.g. leaves) or from short-lived messenger RNA. This method, therefore, does not involve the stable insertion of heritable traits into genomic DNA of the host organism.

Transient expression is a useful research tool that offers greater flexibility, lower costs and rapid results compared to the development of a line of stably transformed plants. It can produce relatively large amounts of recombinant proteins rapidly over a number of days. The nature of the system means that there are some limitations of scale and as such, transient expression methodologies are not always comparable with the type of large-scale production that can be achieved with fields of transgenic plants. It is often been used to verify that gene products are structurally and functionally stable before progression onto large-scale transgenic production (Fischer *et al.* 1999b; Twyman *et al.* 2003). Recent improvements in the technology have enabled production to be scaled up to some extent and have improved yield such that some commercial production is being developed.

There are several approaches to deliver genes into the cells of whole plants. These include biolistic delivery, infiltration with recombinant *Agrobacterium*, and infection with modified viral vectors (Grill *et al.* 2005).

Biolistic delivery inserts segments of DNA into plant cells via particle bombardment. It is a very targeted approach and requires that the DNA segments reach and are encoded into the nucleus for the genes to be expressed. Genes will only reach a small number of cells and as such this method is not suitable for the expression of large amounts of recombinant protein but may be used for limited analysis of protein stability (Christou 1996; Fischer *et al.* 1999b).

Infiltration with recombinant *Agrobacterium* (agroinfiltration) can be used to target many more cells and can be used to induce expression in leaves that have been removed from the plant. The genes of interest are cloned into vectors and inserted into strains of *Agrobacterium*. The bacterial cells are then inserted into the leaf tissues via vacuum infiltration. The *Agrobacterium* vector inserts the new genes directly into the nucleus of the cells via bacterial proteins in the same way as callus cells are transformed for the generation of stably transformed plants (Kapila *et al.* 1996). This system produces rapid results and sufficient quantities of expressed product for characterisation and assessment of protein stability (Fischer *et al.* 1999b).

Gene delivery to the whole plant can also be achieved via inoculation with recombinant viral vectors (Cañizares *et al.* 2005, 2006). The gene of interest is inserted into the genome of a plant virus and this is then used to infect the plant. Once inoculated, the virus will systematically spread throughout the plant, replicating in the cytoplasm and will infect most of the cells. Many transcripts of the gene are therefore formed within the plant resulting in high levels of expression of the protein (Fischer *et al.* 1999b).

High-throughput viral expression systems have been developed with *Nicotiana benthamiana* using the Tobacco Mosaic Virus (TMV) vector (Escobar *et al.* 2003). The efficiency of the system is such that the most time consuming step is the engineering of the viral vector itself. Marillonnet *et al.* (2004) have devised a procedure to increase the efficiency of this stage by using *Agrobacterium* to deliver DNA segments of the viral vector and gene of interest into the plant. The various components then re-assemble once inside the plant removing the need for vector engineering *in vitro*. High yield of recombinant product were then obtained within 3-14 days (Marillonnet *et al.* 2004), and the method has been recently adopted for the high-level expression (1 mg/gm fresh weight) of human growth hormone (Gils *et al.* 2005).

Table 4.7 Large Scale Biology Corporation products generated via viral transient expression

*Follow on biologicals are established products under patent protection that has or will soon expire.

Product type	Product	Description	Development
Therapeutics	Alpha-galactosidase A	Replacement therapy for the metabolic disorder Fabry disease	Preclinical trials
	Lysosomal acid lipase	Reduces fatty acid deposits in vasculature, used to treat atherosclerotic plaques (heart disease)	Preclinical trials
Follow on biologicals*	Aprotinin	Natural protease inhibitor that reduces blood loss during cardiovascular bypass surgery	Product introduced 2004; distributed through Sigma
	Alpha interferon 2a & 2b; G-CSF; Hepatitis B vaccine antigens; cytokinins; growth factors; enzymes; enzyme inhibitors	Various	Various stages of development
Vaccines	Non-Hodgkin's lymphoma personalised cancer vaccines	Customised patient specific therapeutic cancer vaccines	Early stage clinical trials
	Papillomavirus vaccines	Therapeutic vaccines against various forms of animal and human papillomavirus	Preclinical trials
	Parvovirus	Vaccine against infection of companion and agricultural animals	Initial clinical trials

The Large Scale Biology Company (LSBC) in the US has developed viral vectors for the production of recombinant proteins in *Nicotiana* plants and uses this system for the commercial scale production of pharmaceutical proteins (Marillonnet *et al.* 2004). LSBC has several vaccines and therapeutics in preclinical and initial clinical trials, all produced through viral transient expression (Table 4.7).

LSBC are developing personalised cancer vaccines for non-Hodgkin's lymphoma patients using sequences obtained from tumour hybridoma or human tumour biopsy cells. The sequences are cloned into Tobacco Mosaic Virus vectors and these are used to inoculate *N. benthamiana* plants, which then secrete the scFv vaccines (McCormick *et al.* 2003). The rapid turn around of the viral vector system enables patient specific therapeutic vaccines to be produced in 6-10 weeks as opposed to several months by conventional means. These vaccines have completed early stage clinical trials (<http://www.lsbc.com/thera.html>).

LSBC have demonstrated that their TMV vectors are not pollen or seed transmitted and do not pose any containment issues. In addition, LSBC have based their system around *Nicotiana benthamiana* which is more susceptible to TMV than the commercial cultivars of *N. tabacum* that have been bred to confer inherent resistant to TMV. All of LSBC's pharmaceutical and veterinary products are manufactured in controlled greenhouses and growth rooms, although external field trials have been

carried out. Growing the plants indoors offers more environmentally stable conditions, resulting in higher yields and more consistent recovery of recombinant products.

It has been demonstrated that recombinant TMV viruses are not likely to persist in populations of tobacco should they escape confinement. Rabindran and Dawson (2001) examined the progression of transgenic TMV vectors in *N. benthamiana* plants and found that as the virus spread, the transgene was excised and any hybrids that formed between the released and wild-type viruses were poor competitors against the wild type TMV.

Transient expression has also been demonstrated as commercially viable in other leafy crops. Negrouk *et al.* (2005) optimized an agro-infiltration procedure to produce as much as 20-80 mg of functional antibody per kilogram of fresh lettuce leaf tissue in less than a week. This procedure has been used to produce a humanized IgG1 kappa anti-tissue factor antibody (hOAT) as well as other antibodies. The results demonstrate that transient expression of recombinant antibodies in lettuce is very efficient, inexpensive and readily scalable. Thus, it is a useful tool for small-scale production of functionally active antibodies and other pharmaceutically important proteins. In addition, it can be used to facilitate humanization or optimisation of antibodies or to produce quantities of antibody (in the range of 100 mg) sufficient for in vivo studies. Commercialisation of the process has taken place through Altor Bioscience (<http://www.altorbioscience.com/>) who recently (31 July 2005) announced that it had received a Phase I Small Business Innovation Research (SBIR) grant from the National Cancer Institute (NCI) to support development of its proprietary processes for making therapeutic antibodies in transgenic lettuce. In the NCI-supported project, Altor will extend the published project (Negrouk *et al.* 2005) and conduct preclinical efficacy testing in tumour models using antibodies already produced at high levels in stable transgenic lettuce. A related study on lettuce has recently been reported by Joh *et al.* (2005)

Further examples of transient expression in plants are:

Rabies vaccines in spinach and tobacco using viral vectors: Yusibov *et al.* 2002

Human respiratory syncytial virus G-protein in tobacco using alfalfa mosaic virus:
Belanger *et al.* 2000

Despite having addressed the regulatory hurdles and public opposition that accompanies stable, genetic transformation with plants, there remain containment concerns relating to transient expression technologies. Although the plants are untransformed, the vectors used to induce expression in both the *Agrobacterium* and viral based systems are still genetically modified organisms and will require regulation should deliberate release be requested. The use of genetically modified plant viruses will require some containment strategies to be in place to prevent the long-term persistence of a viral strain encoding a pharmaceutical product.

4.2.1 Summary of transient expression

Transient expression enables the production of GM products from non-GM plants. Traits may be introduced into specific tissues of whole plants via:

- **Biolistics**
- ***Agrobacterium***
- **Viruses**

Generates rapid results and is less costly than the development of stable transgenic lines but with some limitation of scale.

The Large Scale Biology Corporation use only transient expression using *Nicotiana benthamiana* and tobacco mosaic virus vectors in physically contained, greenhouse conditions.

Containment of the GM vector is the major regulatory issue with this technology.

4.3 Alternative non-crop species

The adoption of species not conventionally cultivated for food or feed as production platforms for industrial and pharmaceutical proteins offers additional containment reassurances as these products have a minimal chance of entering the food chain. In addition, such species will require the development of specific handling and processing facilities and, depending on the choice of organism, may be grown in physically contained conditions.

4.3.1 Flowering plants

4.3.1.1 Safflower

Safflower (*Carthamus tinctorius*) has been adopted as a production system for biopharming by Syngenta and SemBioSys Genetics Inc (www.sembiosys.com). Safflower is an old crop previously cultivated for dyes and food colouring before cheaper aniline based dyes came on to the market. It is a highly productive oilseed crop and thus only relatively low acreages are required. It is also self-pollinating and SemBioSys claim that virtually no pollen is transported by the wind and that no weedy relatives are found in the Americas.

SemBioSys have developed and patented a recombinant protein production system for safflower, whereby the transgene product is linked to an oleosin fusion protein and is captured on oil bodies in the seed. The harvested seed are milled in an aqueous buffer and centrifuged to separate the oil and aqueous fractions. The oil bodies are then easily removed and the transgene product purified and processed for its downstream application. This StratosomeTM Biologics System enables the recovery of 1Kg recombinant protein from 1 tonne of seed.

SemBioSys have carried out four field trials in the US and 17 in Canada of safflower expressing pharmaceutical proteins or aquaculture feed additives. They have also produced a safflower crop in Mexico and Chile (as stated on their website, www.sembiosys.com). Their main pharmaceutical products are insulin (Nykiforuk *et*

al. 2006) and apolipoprotein AI (a cardiovascular therapy for heart attacks and angina) both of which are in the research phase and planned to enter clinical trials in 2007-8. In addition, SemBioSys are developing an immunostimulatory protein feed additive for shrimp that improves their ability to survive viral infection. This product, ImmunoSphere™ has been successfully tested in tank trials and is due to enter pond trials in 2006. SemBioSys is also developing animal vaccines using their Stratosome™ Biologics System with Dow AgroSciences.

4.3.1.2 *Lemna*

The monocot family Lemnaceae is composed of small, free-floating aquatic plants, the most common of which is duckweed or *Lemna*. *Lemna* is distributed worldwide and has biomass doubling times of 48-72h under controlled, axenic conditions. Reproduction is typically vegetative with culture mass increasing exponentially until crowding sets in. *Lemna*, however, can also flower and produce seeds.

The most commercially advanced species is *Lemna minor* (Gasdaska *et al.* 2003), a fresh water plant used as feed and food, mostly in Asian countries. It is also used in North America for wastewater management.

The rapid reproductive rate and aquatic nature of these plants makes them ideal for bio-pharming in physically contained water-based reactors. A transformation method was developed for *L. minor* several years ago (Yamamoto *et al.* 2001). Two independent companies Biolex (US) (www.biolex.com) and LemnaGene (Lyon, France) (<http://www.lemnagene.com/>) exploited this technology but recently (28 July 2005) the US company acquired its French rival and now has a dominant position.

The system developed by Biolex has been named the LEX System™ (Table 4.8) and this has been developed to produce hard-to-make proteins (such as peptides and cytokines) and monoclonal antibodies. Biolex state that:

‘all the human therapeutic proteins Biolex has attempted (including a peptide, multimeric proteins, alfa interferon and other cytokines, and monoclonal antibodies) have been expressed successfully. Each of the proteins characterised to date has

been demonstrated to have the predicted composition, desired physical structure and correct biological activity’.

Table 4.8 The LEX System™ of bio-pharming

LEX System™ : the ideal system for therapeutic proteins	
Clonal	Genetic stability without pollen or seed
Fast	Doubles biomass every 36 hours
High expression levels	Optimizes scale-up & downstream processing
Secretes target protein	Simplifies downstream processing
Contained and controlled	Conforms with GMP guidelines
Inexpensive	Low operating and capital costs
Scalable	Proprietary multi-ton production format

Compared to conventional mammalian manufacturing plants a LEX facility presents and eight-fold reduction in start up costs. It is stated that a LEX facility can be built for \$50 million in three years, while a mammalian facility can cost as much as \$400 million and may take up to five years due to the control systems needed to prevent contamination.

Biolex holds several patents relating to the transformation of *Lemna* and has continued to develop the LEX System™ over the past five years. The broad patent portfolio relates to the method of transformation, the selection, recovery and growth of the transgenic plants; the composition of expression and enhanced expression through to the development of automated high throughput systems (Table 4.9).

Table 4.9 Patent portfolio of the Biolex LEX System™

Patent Number	Date	Title	Claims made of the invention
US 6040498 WO 9907210	31 March 2000	Genetically engineered duckweed	methods of: transformation and selection; transgenic plant regeneration; growth and recovery; use of multiple genes and vectors; transformed tissue and plants
WO 0210414	7 Feb 2002	Expression of biologically active polypeptides in duckweed	methods and compositions for: expression, recovery; enhanced expression levels; directed secretion.
WO 02097029	Dec 2002	Plant and method for high throughput screening	automation of the LEX System™; commercial line creation technology
US 030033630	Dec 2002	The use of duckweed in high throughput screening	automation of the LEX System™; commercial line creation technology
US 6680200	20 Jan 2004	LED array for illuminating cell well plants and automated rack system for handling the same	automation of the LEX System™; commercial line creation technology

In addition, on May 6, 2004 Biolex acquired Epicyte Pharmaceutical and as such has achieved a dominant intellectual property position via Epicyte's Plantibodies™ patent portfolio. In September 1997, The Scripps Research Institute granted to Epicyte an exclusive, worldwide license to the entire patent estate covering the expression of human and other antibodies in plants. In July 2002, US patent 6417429 was issued, extending the Plantibodies™ patent protection to all species of transgenic plants that produce an antibody and methods of making the transgenic plants. In addition, the Plantibodies™ portfolio includes issued patent claims to plant-made antibody compositions and their methods of use (Table 4.10).

Table 4.10 List of patents issued to Plantibodies™

Patent number	Invention
US 5202422	Composition claims
US 5639947	Ig/Transgenic plants
US 5959177	Secretory antibodies
US 6417429	Ig/Transgenic plants
JP 3238700	Ig/Transgenic plants

Pending Plantibodies™ patents include additional claims to transgenic plants expressing other multimeric proteins, immunization strategies, pharmaceutical compositions and plant-based manufacturing methods. The Plantibodies™ portfolio also has several continuation applications on file. These applications include claims to compositions, transgenic plants, plant cells and methods of production for:

- Single immunoglobulin molecules including single chain antibodies;
- Heteromultimeric proteins not normally produced in plants;
- Immunoglobulin molecules including fragments Fab, Fab', Fv;
- Methods of passive immunization using plant made antibodies.

Based on the issuance of the July 2002 patent, Biolex believe that the Plantibodies™ patent portfolio covers all aspects of producing any antibody in any plant. The most developed of Biolex' products is Locteron™, a novel controlled-release form of a hepatitis C treatment that includes the alpha interferon BLX-883. Locteron™ entered Phase 1 clinical trials on 24 healthy volunteers, in February 2005. On 26 October 2005 Biolex announced the successful completion of the trial of this product which is

the first clinical-stage therapeutic candidate produced in Biolex's GMP facilities using its LEX System(TM). In the trial, BLX-883 was safely administered at a clinically relevant dose and demonstrated bioactivity consistent with Intron® A, a currently marketed alfa interferon used as a comparator. Alfa interferon is used in the treatment of hepatitis C, hepatitis B, and multiple cancers and its worldwide sales currently exceed \$3 billion. The study was conducted under an IND submitted to the United States FDA and a CTA filed with the United Kingdom MHRA. Participants were administered one of three doses of BLX-883. The 12 participants who received the clinically relevant dose of 3 million international units also received the same dose of Intron A to allow for a direct comparison of the two agents. No safety concerns arose during the study, and the side effects attributable to BLX-883 and Intron A were comparable. Bioactivity of the two agents was also compared, and the elevation of specific immune system markers, such as neopterin, measured after administration of BLX-883 was consistent to the elevation measured after administration of Intron A.

More recently, 27th October 2005, it was announced that Biolex and Kringle Pharma had formed a collaboration for the production of a novel protein targeting cancer. The protein, NK4, is an elastase-generated fragment of hepatocyte growth factor (HGF) containing four kringle domains, initially discovered as a competitive antagonist of HGF. Research suggests that it also possesses the anti-angiogenic property relevant to cancer and neovascularisation disorders and thus has potential as a drug therapeutic. Preclinical testing of NK4 has shown inhibition of both metastasis and angiogenesis in multiple animal tumour models. Efficient production of NK4 using the LEX System will accelerate its rapid clinical development and commercialisation.

As with algae, the rapid reproductive rate that makes *Lemna* an attractive production system also raises concerns regarding escape. Physical containment will prevent hybridisation with wild species and enables absolute control over the whole process. However, safeguards must be in place to prevent escape of the organism via wastewater. Any release into the water system of transgenic *Lemna* expressing products into the growth medium would be of great concern due to the nature of the organism and would be very difficult to eradicate without further contamination of the water.

4.3.1.3 *Spirodela*

Spirodela is also a member of the Lemnaceae and as one of the less reduced forms of duckweed is commonly known as giant duckweed, although the plants are still comparatively small (Figure 4.8). *Spirodela* also has a worldwide distribution and the same biomass doubling capacity as *Lemna* (48-72h). Unlike *Lemna*, *Spirodela* rarely produces any flower or seed. It is cultivated in some places in the US on dairy farm wastewater. It has a high protein content ($\pm 50\%$ dry weight), contains a good balance of essential amino acids, and may be used as an alternative for alfalfa in animal feed.

Like *Lemna*, the rapid reproduction capacity of *Spirodela* offers an efficient production system for bio-pharming and as it only reproduces vegetatively, there is no diversion of resources towards the flowering process. *Spirodela oligorrhiza* has been transformed by the Weizmann Institute who have developed stable transformed plants with expression of transgene products ranging from 0.005% to about 3% of total protein. As *Spirodela* is edible, the dried plant material can be used in the final biotech product without the need for a protein extraction and purification stage.

Production takes place in totally enclosed growth facilities (Figure 4.7) thus adopting a physical containment strategy. As with *Lemna*, the greatest risk of escape would appear to be via wastewater from the production system. Treatment of wastewater prior to discharge into the water system would need to be adequate to remove all organisms to prevent accidental release.

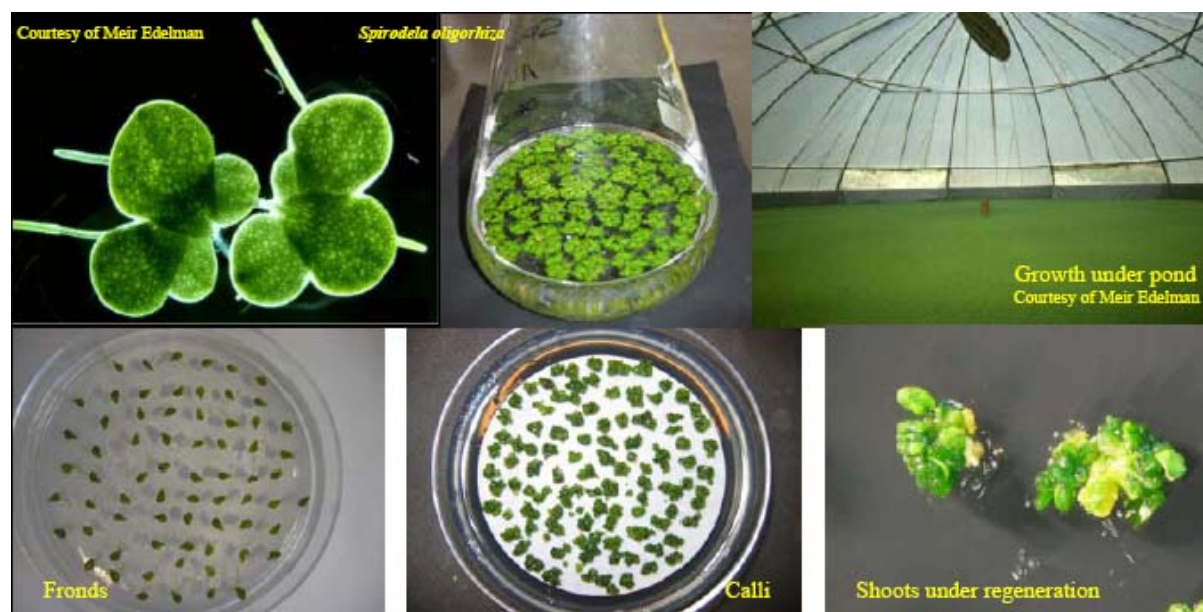


Figure 4.7 Growth of *Spirodela oligorrhiza* in culture.

(Source: <http://www.lemnagene.com/communication/poster.pdf>).

4.3.2 Algae

Algae are a division of plants that inhabit a range of aquatic habitats from marine and fresh water, to damp terrestrial areas. Some algae are single celled, others are more structured, but none possess roots, stems or leaves. Algae reproduce by spores and many species are capable of sexual and non-sexual reproduction i.e. vegetative propagation. The potential of using transgenic algae as a source of novel products, including a range of pharmaceutical proteins, has been reviewed recently (El-Sheekh 2005; Franklin & Mayfield 2004, 2005; Walker *et al.*, 2005). The ease with which algae can be transformed, their short-life cycle from generation to generation, and the fact that algal cultivation requires some degree of physical containment make algae an attractive alternative for the bio-pharming of pharmaceuticals and industrial products. The species involved may be conveniently divided into the single cell forms and the multicellular species.

4.3.2.1 Single celled species

The single-celled alga *Chlamydomonas reinhardtii* has long been used as a model species since it has a small genome (now sequenced) and is readily transformed

(Mayfield *et al.* 2003; Fuhrmann 2004; Leon-Banares *et al.* 2004; Mayfield & Franklin 2005). In addition, the commercial exploitation of other species of non-transgenic algae as a nutritional supplement is relatively well developed (Table 4.8).

The use of transgenic algal systems as bioreactors for industrial and pharmaceutical compounds is also developing (Table 4.11) although it has not yet reached the market place. Single-celled algae offer many benefits compared to higher plants as a bio-pharming system as they have relatively fast growth rates, can be grown in physically contained conditions under high densities, and with the right conditions of light and aeration, can be grown in megalitre volumes (Walker *et al.* 2005). In addition, the simple structure of micro-algae compared to higher plants makes the protein purification process simpler and therefore less costly. The reduced costs compared to higher plants have led to an increase in commercial activity in this sector and the formation of several new biotech companies (Walker *et al.* 2005) (Table 4.11)

Table 4.11 Companies currently selling microalgal-based products or developing microalgae as bioreactors for protein production

Company	Microalgae	Products
Cyanotech (www.cyanotech.com)	<i>Spirulina pacifica</i>	Spirulina extracts as nutritional supplements, immunological diagnostics, aquaculture feed/pigments and food colouring
Martek Biosciences Corp (www.martekbio.com)	<i>Cryptocodinium cohnii</i>	Nutritional fatty acids
Mera Pharmaceuticals (www.aquasearch.com)	<i>Haematococcus pluvialis</i>	Natural astaxanthin as a nutraceutical
Earthrise Nutritionals (www.earthrise.com)	<i>Spirulina sp</i>	Nutritional supplement to inhibit replication and infectivity of viruses including HIV, CMV, HSV and influenza
PharmaMar (www.pharmamar.com)	Various	Anticancer drugs derived from marine microorganisms
Nikken Sohonsa Corp. (www.chlostanin.co.jp)	<i>Chlorella</i> spp.	Dietary supplements: polysaccharide
	<i>Dunaliella</i> spp.	N,β-1.3 glucan and β-carotene
Nature Beta Technologies	<i>Dunaliella bardowil</i>	β-carotene powder
Cognis (www.cognis.com)	<i>Dunaliella salina</i>	Mixed carotenoids
Subitec GmbH (www.subitec.com)	Undisclosed	Polyunsaturated fatty acids
Solazyme (www.solazyme.com)	Undisclosed	No products yet brought to market
Phycotransgenics (www.phycotransgenics.com)	<i>Chlamydomonas reinhardtii</i>	Transgenic microalgae for animal health/feed, bioremediation, environmental monitoring and biopesticides
Algal Biotechnology (www.algalbiotechnology.com)	<i>Chlamydomonas reinhardtii</i>	Developing recombinant protein technology

In July 2000, Martek Biosciences (<http://www.martekbio.com/home.asp>) of Columbia, Maryland were granted US Patent 6027900 (Methods and Tools for Transformation of Eukaryotic Algae). This patent outlines a process to express non-algal genes in algal cells in order to generate key pharmaceutical and industrial compounds within algal bioreactors. The company has an extensive interest in algal derived food supplements including docosahexaenoic acid, a baby food ingredient claimed to aid mental development.

The necessary production facilities for microalgal growth are well established as shown in (Figure 4.8-4.9).

The most topical example of exploiting transgenic algae is the application to grow large quantities of transgenic *Chlamydomonas* in Hawaii (<http://www.isis.org.uk/PICGA.php>). The details of this specific case are as follows. On 1 November 2004, the Hawaii Department of Agriculture received an application from Mera Pharmaceuticals (<http://www.aquasearch.com/>) (in collaboration with Rincon Pharmaceuticals, <http://www.rinconpharma.com/Docs/Mera-Rincon%20PR%20050513.pdf>), for a permit to begin large-scale production of a genetically modified alga, *C. reinhardtii*, producing a human immunoglobulin-A protein active against a variant of the herpes simplex virus. On 17 April 2005, Mera Pharmaceuticals revised their application to include seven additional GM strains expressing a range of human antibodies, interleukins and nerve growth factors.

The details of the 8 strains involved are:

1. Strain Hsv8, producing a full-length human immunoglobulin-A against a variant of the herpes simplex virus;
2. Strain aFceR 1r-1, producing a protein targeting the Fc portion of the IgE molecule, thereby limiting the interaction between circulating IgE molecules and receptors on mast cells, which in turn limits the release of histamines and reduces inflammation;
3. Strain aTNFr-1, producing IgG1 anti-tumour necrosis factor antibody;
4. Strain a TNr-1, producing IgG1 anti-microbial antibody;
5. Strain aCRr-1, producing IgG1d anti-cell proliferation antibody;



Figure 4.8 Microalgae *Haematococcus pluvialis* as seen under the microscope
(Source: <http://www.biopro.de/en/stern/magazin/01402/>)



Figure 4.9 Algal Photobioreactor
(Source: <http://www.biopro.de/en/stern/magazin/01402/>)

6. Strain aBSSsr-1, producing anti-cancer cell specific antibody;
7. Strain aLL 10r-1, producing various interleukins (including interleukin 10, interleukin 13, interleukin 5 and interleukin 3);
8. Strain aARTr-1, producing neurotrophic factors to stimulate the growth of new nerve tissue.

The case for the first GM strain was heard on 25 May 2005, and the Plant Quarantine Honolulu Office did not grant the permit. The application for the seven other GM strains had been scheduled for 7 October 2005, but was brought forward to 29 June. On that occasion, the state Board of Agriculture gave its approval. Mera Pharmaceuticals will begin the process of importing the microalgae from The Scripps Institute, La Jolla, California, though some opponents are seeking to block the permit. On 3 August 2005, a lawsuit was filed against the state Board of Agriculture challenging the agency's approval of a permit to allow the production of GM microorganisms on the Big Island. Four citizen groups argue that before the algae are grown to produce pharmaceuticals on the island of Hawaii, an environmental assessment should be conducted. The groups 'Ohana Pale Ke Ao and Kohanaiki 'Ohana, as well as GMO Free Hawaii, and the Sierra Club, Hawaii Chapter, represented by Earthjustice, are claiming that the organisms are potentially dangerous. On 10 October 2005, the court upheld the complaint and ruled that an environmental assessment must be conducted before the project can proceed.

It was intended that the algae be imported into Kona on the island of Hawaii to be grown in the outdoor bioreactor system at Keahole Point at the state's aquaculture park Nelha. This type of large-scale trial of a biopharmaceutical alga has never been conducted before in the US, and all government agencies, the Food and Drug Administration (FDA), the US Department of Agriculture (USDA) and Environment Protection Agency (EPA) have waived oversight of the trial. The commercial support for the project is continuing with additional funding of \$4.7m being recently announced by Rincon Pharmaceuticals who have exclusive rights to a number of patents (US 6156517, 6294653) and a patent application (US 040014174) on relevant technologies developed at the Scripps Research Institute.

In a related area, The Department of Commerce (DOC), National Oceanic and Atmospheric Administration (NOAA) made an award in 2001 through the Small Business Innovation Research (SBIR) programme to Phycotransgenics LLC (<http://www.phycotransgenics.com/>) to develop a fish vaccine using transgenic *Chlamydomonas*. They have also received funding for similar project on poultry vaccines. Similar studies on the use of GM plants (potato tubers, see section 4.1.7) as a production system for fish vaccines (Companjen *et al.* 2005) are being conducted by the Fishov consortium (<http://www.fishov.org/>), an EU project that commenced on 1st Jan 2002 and coordinated from Plant Research International, Wageningen, Holland. The specific targets of this project are viral haemorrhagic septicaemia and spring viraemia, devastating diseases of salmonids and carp respectively.

Other studies involving transgenic unicellular algae include expression of cholera toxin B subunit expressed in *Chlamydomonas* (Sun *et al.* 2003) and the hepatitis B surface antigen (HBsAg) gene in *Dunaliella salina* (Geng *et al.* 2004). In addition, transformation systems for other algae are being developed. For example, the completion of the *Thalassiosira pseudonana* genome project has led to the development of transgenic diatoms (Poulsen & Kröger 2005). However, only few species of microalgae can as yet be routinely transformed (Walker *et al.* 2005) but this is expected to rise as the industry develops.

Many of the features that make single celled algae an attractive bio-pharma organism, such as a short generation time and relatively high growth rates, also raise concerns about escape. Algae are natural colonisers and some species have a tremendous capacity to disperse and persist in natural habitats. As yet, there is no feasible method of genetically controlling reproduction in algae and a basic lack of understanding of the biology of algae is considered by some to be a major impediment to the development of engineered control of spread (Anon 2004). As such, physical separation remains the only confinement measure available to prevent transgene escape.

4.3.2.2 Multicellular species

Multicellular algae include filamentous and parenchymatous or thalloid forms such as seaweed. At present, there is no commercial production of transgenic seaweed but there is increasing interest in its potential to produce fuels, polysaccharides, fish feed, and pharmaceutical products and for use in bioremediation (Anon 2004). Genetic transformation systems have been developed for several seaweed species including the red seaweeds *Porphyra*, *Gracilaria*, *Grateloupia*, *Kappaphycus* and *Ceramium*, the green seaweed *Ulva*, and the most commonly cultivated seaweed, the brown seaweed *Laminaria japonica* (kelp) (Qin *et al.* 1999, 2005). The majority of this development work has been carried out in China and a Chinese Patent covering the transformation of kelp (96120235 Method of producing transgenic kelp. 23 Oct 1996) has been issued (Qin Song *et al.*).

Expression of pharmaceutical products has been proven in kelp by the insertion of a heterologous gene encoding hepatitis B surface antigen (HBsAg) via particle bombardment. Results of quantitative ELISA showed that HBsAg in this material was 0.529 µg/mg soluble proteins on average and the highest value was 2.497 µg/mg, implying that recombinant HBsAg had the natural epitope (Jiang *et al.* 2002).

In the United States, the Northeastern University has filed a patent application (WO 0062601) relating to transformation of multicellular marine algae via *Agrobacterium*. The application describes transformation of the red algae *Porphyra*, widely used as a staple food in Japan known as nori, for which worldwide production is estimated at \$1.5 billion dollars annually.

A method of confinement described as 'biological design' has been used to contain invasive species of *Porphyra* cultivated in Maine in the US. A non-transformed, non-native species *P. umbilicalis* was introduced and cultivated commercially on floating drafts in coastal waters inhabited by a closely related native species. Fears that the introduced invasive species would hybridise with and out-compete the native species were allayed by extensive fieldwork demonstrating that the introduced species was not invasive outside its native range. The data revealed that the introduced species

was unable to reproduce presumably due to its inability to survive the winter conditions (Levine *et al.* 2001). The species was therefore considered to be biologically confined (Anon 2004).

Genetic containment strategies are not developed for algae due to a lack of knowledge of their biology and reproduction, and the complex life histories that many algal species possess. Algae demonstrate multiple reproductive pathways including parthenogenesis (the autonomous development of an egg cell into an embryo without fertilisation) and the production of vegetative propagules. At the current level of understanding, containment strategies for GM algae need to be considered on a case by case basis.

4.3.3 Moss

The predominant moss species used for bio-pharming purposes is *Physcomitrella patens*. This species is a model organism and has been used in genetic studies for over 30 years. The genome is small and is currently being sequenced (<http://www.jgi.doe.gov/sequencing/why/CSP2005/physcomitrella.html>).

There are several academic and commercial groups working on *Physcomitrella* as a bio-pharming system (Decker & Reski 2004; Hohe & Reski 2005). *Physcomitrella* is unique in that it is the only known plant that can be engineered by a targeted gene disruption method known as homologous recombination. This method allows the removal of any unwanted side effects as a result of transformation and the elimination of any unwanted gene function. This means that one of the major drawbacks of producing human proteins in plants, namely allergic reactions caused by plant-specific glycosylation, can be diminished by targeted knockout of the responsible genes in moss and the consequential alteration of the glycosylation pattern to mimic that of mammalian cells. The system offers a real alternative to mammalian cell lines or transgenic animals for the production of recombinant proteins (Huether *et al.* 2005; Koprivova *et al.* 2003, 2004). In addition, moss cells have a high level of productivity in culture (Hohe *et al.* 2002a). In recent developments of the system, enhanced recovery of a secreted recombinant human

growth factor was achieved by co-expression of human serum albumin (Baur *et al.* 2005a,b), and a conditional heat-shock promoter has been developed (Saidi *et al.* 2005).

The commercial exploitation of this species is the focus of greenovation BioTech GmbH (<http://www.greenovation.com/>) in Freiburg (<http://www.bio-pro.de/en/region/freiburg/magazin/01404/index.html>). The moss is reproduced vegetatively and maintained in the juvenile protonema stage by mechanical disruption. The protonema, the filamentous plant growth stage produced from the spore (Figure 4.10), are cultivated under photoautotrophic conditions in a medium consisting of water and minerals. This currently takes place in 30-litre moss reactors but greenovation are expanding this to 1000 l. The moss grows rapidly and secretes the desired protein into the medium (Figure 4.10) making harvest and purification of the protein from the liquid medium relatively simple. Amongst the compounds produced is the blood coagulation factor IX. The whole process takes place in bioreactors under controlled conditions thus offering a physical containment strategy.

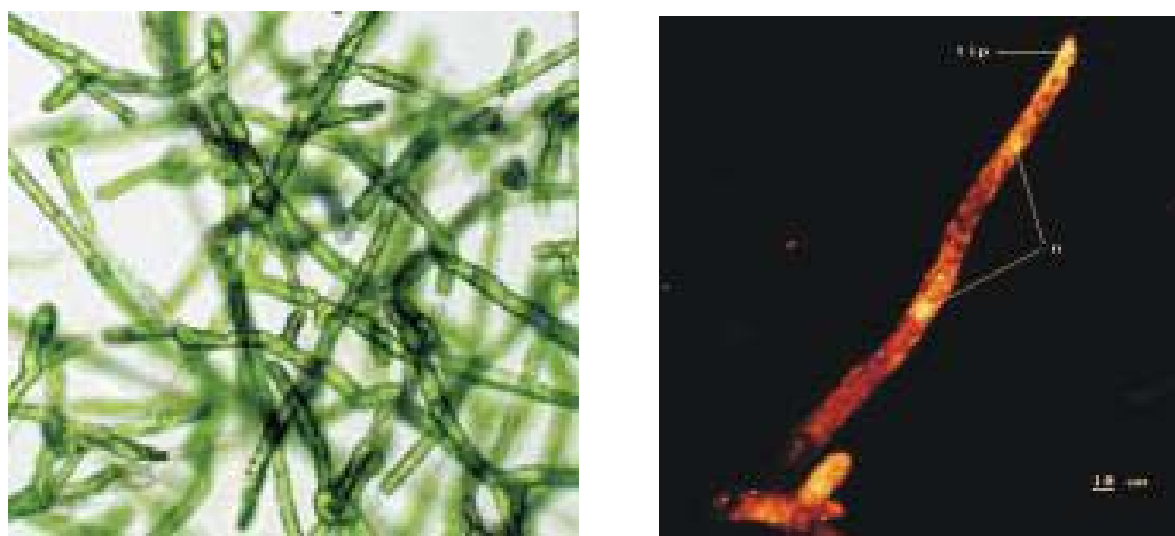


Figure 4.10 Protonema of *Physcomitrella patens* (left). Protein (yellow) is released into the medium (right).

(Source: greenovation)

Greenovation are currently working in collaboration with The Dow Chemical Company to develop glycosylation patterns in *Physcomitrella* for the expression of human pharmaceutical products.

As with algae, mosses have a complex reproductive strategy that is unlike seed plants and require free water to transport the male gametes during sexual reproduction but dry conditions for spore dispersal. Genes controlling sexual reproduction in *Physcomitrella* have not yet been identified, but research into the physiological regulation is revealing ways in which it can be controlled (Hohe *et al.* 2002b). It can be assumed that once the genes have been isolated, the genetic control of spore dispersal and sexual reproduction in *Physcomitrella* will be relatively simple to engineer. Such a development may allow the scale-up of GM moss from bioreactors (Figure 4.11) to larger scale external methods of cultivation.



Figure 4.11 Tube reactor filled with protein-producing moss
(Source: greenovation)

4.3.4 Summary of non-crop species production platforms

Safflower has been adopted as a production platform for novel proteins by some companies:

- productive oilseed crop
- no wind borne pollen
- no wild relatives in the Americas

Pharmaceutical and industrial products can be generated in physically contained, water based bioreactors of simple plants:

- Algae
- Moss
- *Lemna* and *Spirodela* (duckweeds)

Genetic containment strategies have not been developed for these plants.

4.4 Other protein expression techniques

The use of plant cells or specific parts of plants in bio-pharming enables strict physical containment to be employed and removes the need to express the transgene in the reproductive and/or vegetative parts of the plant. Therefore, the risk of transgene escape or entry into the food chain is minimised.

4.4.1 Suspension and callus cell cultures

Cell lines of several species have been used for pharmaceutical production. (Xiao *et al.* 2003). For example, tobacco cell lines have been used for the production of:

Heavy chain immunoglobulin G: Shin *et al.* 2003a

Hepatitis B surface antigen: Kumar *et al.* 2003, Smith *et al.* 2002; Sojikul *et al.* 2003

Human erythropoietin: Matsumoto *et al.* 1995

Human granulocyte-macrophage colony-stimulating factor (GM-CSF): Bodeutsch *et al.* 2001; James *et al.* 2000; Kim *et al.* 2004; Kwon *et al.* 2003b; Lee *et al.* 2004

Human interleukin-2 and interleukin-4: Magnuson *et al.* 1998

Human lactoferrin: Choi *et al.* 2003

Human milk protein sCD14: Girard *et al.* 2004

Human tissue transglutaminase: Sorrentino *et al.* 2005

Mouse monoclonal antibody heavy chain gamma: Magnuson *et al.* 1996

Soluble mouse single-chain antibody, scFv: Xu *et al.* 2002

TMV-specific full-size murine IgG-2b/kappa-antibody: Fischer *et al.* 1999a

Recombinant human granulocyte-macrophage colony stimulating factor (hGM-CSF) (Shin *et al.* 2003b, Hong *et al.* 2005) and human $\alpha(1)$ -antitrypsin (Trexler *et al.* 2003; 2005) have also been produced in rice cell suspension cultures. Most recently, a novel system was reported for the production of somatic embryos of Siberian ginseng (*Eleutherococcus senticosus*), expressing *Escherichia coli* enterotoxin B subunit (Kang *et al.* 2005a). This involved the use of 130 litre air-lift bioreactors. A much larger industrial scale production using 10 ton reactors is already in place for the production of ginseng (CBN Biotech, Korea) and such a system could presumably be adopted for the production of higher value GM compounds.

The adoption of cell culture technology on a commercial scale progressed in February 2004 (<http://news.dow.com/prodbus/2004/20040217a.htm>) with the announcement from The Dow Chemical Company and NOBEX Corporation (<http://www.nobexcorp.com/index.html>), a drug development company specializing in drug delivery, that they will collaborate on plant-based production of NLC-001, a proprietary peptide currently in preclinical development by NOBEX as a potential appetite suppressant to treat obesity.

This project broadens the scope of Dow Plant Biopharmaceuticals beyond their current work in plant-based pharmaceutical production, which has focused primarily on antibodies. Under the agreement, the initial expression of the peptide will take place in plant cell suspension to allow for fast evaluation of the ability of plant tissues to secrete the peptide. If successful, production may be moved to whole plants for rapid scale-up.

More recently (7 June 2004) The Dow Chemical Company acquired a license to certain patents and patent applications from Epicyte Pharmaceutical, Inc. The license is related to the expression of antibodies and other immunoglobulin-derived molecules in plants.

4.4.2 Root culture, hairy roots and root exudates

The transformation of tobacco plants to secrete products in their roots is described in Borisjuk *et al.* (1999), Drake *et al.* (2003), and Tesfaye *et al.* (2005). This approach engineers the plants to produce recombinant proteins and secrete these products (Vitale & Pedrazzini 2005) from their roots into medium.

This technology was first used by Phytomedics, in Dayton, New Jersey, which later absorbed Photosynthetic Harvest Inc. (Willingboro, New Jersey). On August 3 2004 they announced a joint venture with ARC Ventures Inc who specialise in the production of hydroponically raised vegetables and herbs, to form Phytomedicinal Greenhouses LLC (PMG). The aim of PMG was to develop industrial scale production platforms of hydroponically maintained transgenic plants to produce a

range of recombinant products from pharmaceuticals to food supplements. PMG developed their 'recombinant protein secretion technology, REPOST™ as a bio-manufacturing process based on the secretion of target protein from hydroponically cultivated roots.

The process can be used to produce relatively pure recombinant proteins that are shown to retain their biological activity and are accumulated in higher amounts than in root tissue. In addition, the exudates have the potential to be used to influence soil microbes and pathogens that may infect the plant via the rhizosphere, modify soil nutrients near the plant roots and also may be used in the neutralisation of environmental pollutants (Borisjuk *et al.* 1999; Drake *et al.* 2003; Tesfaye *et al.* 2005).

In a linked study, Gaume *et al.* (2003) infected transgenic tobacco plants with *Agrobacterium rhizogenes* to induce hairy root disease (Figure 4.15). The expansion in root surface area that resulted from the proliferation of root growth led to an increase in the secretion of the transgenically expressed product (Gaume *et al.* 2003).

If single cells are infected with *Agrobacterium rhizogenes*, the induced root growth is genetically stable and of clonal origin. If the cell of origin is transformed to produce a recombinant protein, the product will be secreted from the root mass into the supporting media. For this reason, production systems can be developed and can be used immediately, without going through several generations of self-crossing and selection, and without the use of antibiotic or herbicide resistance markers. Furthermore, recombinant proteins and peptides can be engineered to be secreted and can be continuously extracted from the medium. If it is not possible to target the proteins for secretion, conventional extraction from hairy roots is made easier due to the absence of waxes and pigment molecules that are present in leaves.

This procedure has allowed the production of a full-length murine IgG monoclonal antibody from root culture. Similar procedures have been adopted elsewhere (Gaume *et al.* 2003; Komarnytsky *et al.* 2004; Zhang *et al.* 2005) using root tissue on transgenic tobacco plants engineered to secrete a model recombinant protein and

human secreted alkaline phosphatase. This technology is reviewed by Medina-Bolivar and Cramer (2004) and the problems of the stability of antibodies secreted from roots has been discussed recently (Doran 2005).

The most commercially advanced hairy root culture system is ROOTec (<http://www.rootec.com/english/technology.html>) a company that has developed a bioreactor technology for several species using hairy root cultures generated infecting tissues with the soil bacterium *Agrobacterium rhizogenes* (Figure 4.12) (<http://www.bio-pro.de/en/region/rhein/magazin/01440/index.html>). This can be used for many applications including the manufacture of bioactive compounds that are naturally produced by the roots, for seeking new compounds (biotransformations), and for the production of recombinant proteins in molecular farming. A new company, ROOTec bioactives GmbH, has recently been started in Basel, Switzerland.



Figure 4.12 Fermentors for the production of hairy roots
(Source: www.rootec.com)

4.4.3 Guttation fluid

Guttation is the loss of water through the leaves of plants. It has long been known that some proteins are naturally secreted into the guttation fluid. The rate of guttation

can be increased under controlled conditions of high water absorption, high root pressure and a reduced rate of transpiration.

As a production system, guttation fluid can be collected throughout a plant's life, thus providing a continuous and non-destructive system for recombinant protein production. This has the potential of increasing the efficiency of recombinant protein production technology by increasing yield, abolishing extraction, and simplifying its downstream processing.

Komarnytsky *et al.* (2000) used endoplasmic reticulum signal peptides fused to the recombinant protein sequences to generate transgenic tobacco cv. Wisconsin plants that secrete three heterologous proteins. These proteins were all of different genetic backgrounds and included bacterial xylanase, green fluorescent protein of jellyfish (*Aequorea victoria*), and human placental alkaline phosphatase. Secretion occurs through the leaf intercellular space into tobacco guttation fluid. Production rates of 1.1 µg/g of leaf dry weight per day were achieved for alkaline phosphatase with this protein comprising almost 3% of total soluble protein in the guttation fluid (Komarnytsky *et al.* 2000).

There appear to be no further published studies on the use of guttation fluid as a means of recombinant protein production, but this and the above cell and root culture technologies provide a continuous and non-destructive method of protein production in a physically contained and controlled environment. The development of guttation fluid as a means of commercial production requires further development at this point (Komarnytsky *et al.* 2000).

4.4.4 Summary of other techniques for protein expression

Cell culture expression can be carried out in physically contained conditions.

Product can be collected in solution without the need to harvest the plant:

- **plant suspension and callus cell cultures**
 - **used to express a wide range of products**
- **hairy root cultures and root exudation**
 - **secretion of recombinant product from roots of GM plants or root cultures derived from somatic cells into hydroponic media**
- **guttation fluid**
 - **secretion of recombinant product from the leaves of GM plants**

These technologies remain in the developmental stage.

5. Conclusions

Of the top twenty field crops of Europe, only two do not have GM varieties that have reached the field trials stage of development. GM varieties are being trialled for many European field crops, fodder and forage crops, forestry and fruit trees, grasses and ornamentals. The initial focus of GM technology from herbicide tolerance and insect resistance is now expanding into disease resistance, improved product traits and tolerance to abiotic stress. The potential of these new crop varieties to improve yield and productivity is great. As the number and variety of introduced genes increases, the potential conferred advantage of wild relatives that may acquire transgenes through hybridisation becomes more complex.

Most European crops are capable of crop-to-crop gene exchange and may also be capable of hybridisation with wild relatives in the European flora. For this reason, the need for containment strategies for each GM crops should be assessed on a case by case basis.

With the expansion of GM technology into new countries and new crops, containment of transgenes becomes an increasingly important issue. More research resources are therefore being focused towards devising reliable mechanisms to address this issue. As a result there are an increasing number of containment systems under development for GM crops, many of which at present require additional development time to reach the market place.

Transgene Containment

Physical containment is the simplest form of containment but has obvious restrictions in terms of scale and applicability. In addition, the regulation of underground growth facilities requires clarification in the long term.

Biological containment of transgenes requires that gene flow via pollen and seed from a GM crop is prevented. Preventing transgene escape via pollen can be achieved through plastid transformation and by the induction of male sterility. The insertion of the transgene into the plastid genome is well developed as a containment strategy in tobacco crops and has been adopted for many traits in this

species, including the expression of pharmaceuticals. This technology has been adopted by some sectors of the biotech industry as a platform for pharmaceutical production (e.g. Chlorogen, Inc in the US and Icon Genetics, based in the US and Germany). The expansion of plastid transformation into other crops is at present slowed by incomplete sequence information for chloroplasts of many other species. As data become available, it can be assumed that plastid transformation will become an option for an increasing number of crops. However, for this containment strategy to function as desired, the assumption that all plastids are maternally inherited will need to be verified for each crop adopting this technology.

In contrast, genetically modified male sterility has been achieved in a wide range of crop species by the manipulation of several different development pathways during normal flower development. Knowledge and expertise in plant reproduction and sterility systems have long been used by plant breeders to assist in the generation of hybrid seed. Thus, engineered male sterility systems have developed from an existing knowledge base and are currently adopted in a wide range of crop species including some varieties that are commercially available. However, some limitations exist with the applicability of male sterility as a containment strategy where the fruit or seed is the harvested crop. Such crops may require the presence of a non-GM pollen donor line.

The removal of the transgene from pollen-mediated gene flow does not provide absolute containment however, and seed spillage still offers routes for transgene escape. Indeed, the formation of feral GM crop populations from spilt seed not only offers further potential for transgene escape to wild populations and non-GM crops, but these plants may also become a weed problem if populations are self-perpetuating.

The only containment strategy at present that offers containment via seed is the seed lethality ('terminator') technology. Seed from crops conferred with seed lethality genes will not germinate whether they have developed on the crop, or are the result of crop-to-wild relative hybridisation. The negative public perception of this technology has prevented its development to commercial applications, however and it has not been trialled on a field scale.

Alternative containment strategies based on seed lethality are under development. The recoverable block of function approach attempts to remove the negative aspects of seed lethality by adding an inducible control over the expression of the lethality gene. In addition, new methodologies using complex gene constructs, involving the *trans*-slicing of inteins, controlled excision of the transgene and the insertion of genes to reduce hybrid fitness require further development at present.

Mechanisms to control transgene expression via conditional lethality and inducible promoters have been developed. Such systems either prevent floral or seed development or control the expression of the transgene to limited periods of time. The use of chemical application onto a field of GM plants present a new set of issues for the regulators as deliberate release of a new chemical compound will require assessment. In addition, application will need to be complete across all plants in the field for the treatment to be 100% effective. This has not yet been demonstrated in the field for either of these two containment mechanisms.

The development of 'naturally occurring' containment strategies such as apomixis and cleistogamy is limited at present, as the genes controlling and regulating these conditions remain enigmatic.

Absolute transgene containment is difficult to guarantee with the methodologies available at present and the adoption of any genetic containment strategy will require continuous assessment and monitoring. The separation of transgenes conferring the desired trait(s) and containment genes during chromosomal recombination remains a possibility in the majority of the strategies available and of those under development. The scale of the agricultural production system provides innumerable opportunities for mutation and recombination during a single rotation, therefore monitoring strategies must be rigorous and comprehensive.

Bio-pharmaceuticals

The use of plants to produce industrial and pharmaceutical compounds offers many benefits compared to conventional mammalian cell culture techniques. These benefits include reduced productions costs, increased scalability and the removal of potential contamination from animal disease. This sector is predicted to expand by over 100% in the coming five years and the World Health Organisation is investigating the use of plant based production systems for vaccines and antibodies.

There are a number of plant-derived products already available in Europe. These products are mainly diagnostic and laboratory reagents with a relatively limited market. These have been raised in crops in the US under strict regulation by APHIS using large isolation distances, constant monitoring programmes, and the safe disposal of all plant tissues following harvest. Despite predicted growth in the bio-pharming sector, there are no plans to deregulate any crops in the US expressing recombinant proteins and all crops will continue to be grown under licence from APHIS.

In contrast to the GM plant derived products already commercially available, the second wave of plant-derived products include a range of human vaccines and antibodies. These pharmaceutical proteins are being developed to prevent and treat a wide range of human conditions (tooth decay, cystic fibrosis, diarrhoea, gastrointestinal infection, hepatitis B, Non-Hodgkin's lymphoma, Norwalk virus, pancreatitis, rabies, and vitamin B12 deficiency) and are currently in Phase I and Phase II clinical trials.

Production platforms are being developed for an even wider range of products including insulin (Farinas *et al.* 2005b; Nykiforuk *et al.* 2005), human and animal vaccines (Dus Santos & Wigdorovitz 2005; Lamphear *et al.* 2005; Streatfield 2005) and dietary supplements (Drakakaki *et al.* 2005; Haas *et al.* 2005).

As the plant itself is not the desired end product, production platforms are more flexible and containment issues may be more easily addressed. Tobacco has so far been most widely adopted due to the presence of extensive scientific knowledge in this crop. However, alternative non-crop species are being adopted to prevent contamination of conventional crops with transgenes expressing pharmaceutical products. This includes the use of algae, moss and duckweed species in contained water-based facilities or the use of greenhouse based crops that do not require flowering (lettuce, alfalfa, potato). Indeed, whole plants may not be required and products can be expressed in cell or organ culture.

Considerable success has been achieved with the use of transient expression as a commercially adopted production platform by the Large Scale Biology Corporation using *Nicotiana benthamiana* and viral vectors. LSBC have several products in pre-clinical and clinical trials and have utilised the rapid turnaround time of transient

expression to generate personalised cancer vaccines for non-Hodgkin's lymphoma patients over a much shorter timescale than by conventional means. [Note added in proof: LSBC ceased trading on 23 December, 2005.]

The scale of transient expression technology currently allows crops to be raised in contained growth facilities. However, movement of this technology to the field will require regulation of the GM vector and not the plant production platform.

Issues for the future

The containment of GM technology requires continued development, assessment and regulation to ensure all avenues of transgene escape have been considered and addressed. As no single containment strategy can at present guarantee transgene containment, it is likely that a number of strategies will be adopted to reduce escape potential to a minimum. The adoption of any containment strategy will require constant assessment and monitoring to ensure that any breakdown in the mechanism is picked up and acted upon.

As GM technology continues to develop and innovate, regulatory oversight must continue to move forwards. The adoption of techniques and technologies by industry that require 'less regulation' must be assessed for their environmental impact and risk of transgene escape. The use of contained facilities such as greenhouses, polytunnels and underground mines requires the development and use of standard protocols to ensure that outputs from these facilities, such as biological waste and waste water, contain no living transgenic organism or propagule and pose no environmental risk. The use of lower plants such as algae and mosses in contained facilities should be investigated in the light of their potential for development as pharmaceutical production systems. The factors that make them an attractive production system (fast replication, ease of cultivation) also increase their potential to cause damage if they are accidentally released into the environment. Equally the use of transgenic viral vectors to induce recombinant protein production in non-GM plants in transient expression systems requires assessment. The escape of GM plant viruses from containment has the potential to damage crops and wild plants and may lead to unwanted ecological change.

The next generation of GM crop currently under field trials confer resistance to abiotic stresses such as drought and salt. These crops will allow the cultivation of

areas currently unsuitable for crop production and will offer great potential improving the agricultural output of marginal lands, but also present a new set of issues for containment. For example, areas of land where soils are dry or have a high salt content are inhabited by native non-GM plants and have developed resistance to these conditions and form part of a specific ecosystem. In the event that crop plants or transgenes escape that are also resistant to such conditions, the native plants are likely to be poor competitors in comparison and as such, feral crop plants are likely to become invasive. The potential impact of crop plants resistant to abiotic stresses therefore requires assessment and containment strategies introduced to prevent unwanted ecological change from their introduction.

Seed escape during the sowing of crops and at harvest can lead to the establishment of feral populations of crop plants. Should these crops be GM, seed escape cannot be tolerated. A review of farming practice may identify issues that can be improved upon to reduce and prevent seed escape. Feral populations of non-GM crop plants may be less of a concern, but if they are persistent these plants offer gene flow routes for transgenes from GM plants via cross-pollination. This may occur as crop-feral pollination whereby the feral plant develops seed conferring the GM trait, or as feral-crop pollination whereby the crop is pollinated by the feral plant, which may lead to a breakdown in the containment strategy of the GM crop dependent on pollination from another GM crop plant.

The extent to which seed mediated gene flow is an issue for European crops is not well quantified. An assessment of the relative importance of seed mediated and pollen mediated gene flow would allow containment strategies and farming practices to be tailored to improve the containment of GM varieties. It is likely that this will be different crop to crop and will need to be studied on a case by case basis with due consideration given to the composition of the local flora in areas of cultivation to identify wild plants that may also offer potential for gene flow.

The development of a landscape scale system of testing and monitoring of crops in the field may also be required in the future to ensure that any breakdown in biological containment mechanisms are operating efficiently over a large area. All transgenes, whether for herbicide tolerance, protein expression or containment require extensive field trialling to ensure their long-term stability and function. For example, gene silencing is known to occur via changes in the natural methylation

patterns of plants, especially for genes that have a high physiological cost. Should transgenes be turned off in such a manner it is important to be able to detect this at the field-trial stage so that appropriate action be taken to ensure containment.

The rapid developments in the use of GM plants and transient expression in the production of pharmaceutical and industrial proteins offers great potential for the industry and viable alternative to mammalian-based production systems. The development of new technologies will require regulatory procedures to be developed and applied to ensure that these technologies can be developed in a safe manner to their full potential. It is therefore important that all aspects of transgene escape and environmental impact are addressed whilst these technologies are under development. In the light of this review, a list of recommendations for the development and implementation of containment of GM is given.

Overall Summary of Conclusions and Recommendations

In the short term, a review of the regulatory oversight of plants grown in physical containment, such as glasshouses, polytunnels and underground facilities is required to ensure that best practice is adopted to reduce the risk of transgene escape. This should include an assessment of the use of lower plants such as moss and algae expressing pharmaceutical proteins and contained transient expression systems, where viral vectors are used to induce transgene expression in non-GM plants. The main areas of concern in these production systems being the escape of transgenic algae or plant viruses.

In the longer term, scientific investigations are required to develop a landscape scale system for the testing and monitoring of genetic containment systems. This could include investigations into the effects of gene silencing via methylation and how this may affect the long term stability of transgenes expressing the desired products and of transgenes used to control gene escape. Issues of seed spillage during sowing and harvest should also be investigated with regard to the formation and persistence of feral crop populations. Improvement of farming practice to reduce seed spillage will reduce the formation of feral populations and lessen the opportunities for transgene escape via seed. Persistent feral populations and compatible wild populations allow an escape route for transgenes via pollen mediated gene flow

either by crop-wild or wild-crop pollination. The impact of crop-wild/feral pollination on male sterile crops also requires investigation. An assessment of the relative importance of seed versus pollen-mediated gene flow will aid the targeting of containment strategies to have the greatest impact.

In addition, an environmental impact assessment is required to assess the new generation of GM crops resistant to abiotic stress. Should they escape, these crops have the potential to become invasive and out-compete plants in delicate ecosystems, leading to unwanted environmental change.

Recommendations

1. Assess the thoroughness of the regulatory oversight of GM plants grown in physically contained conditions, including glasshouses, polytunnels and underground mines (section 3.1)
2. Conduct a detailed environmental risk assessment of the use of algae and other lower plants as production platforms for high value and pharmaceutical products (section 4.3)
3. Assess the environmental impact of the next generation of GM crops with conferred resistance to abiotic stress factors (section 1.1)
4. Commission analysis of the application of transient transformation systems, including those using viral vector systems for the production of high value and pharmaceutical products (section 4.2)
5. Investigate the persistence of feral populations of crop plants and maternal based introgression into populations of wild relatives and fields of crops plants from persistent feral populations (sections 1.2, 2.3, 3.2, 3.3)
6. Develop a robust technique for the assessment of genetic and environmental factors affecting gene transfer via plastids in pollen (section 3.2.2)
7. Investigate the relative importance of seed versus pollen mediated gene exchange between crop wild relatives (sections 1.2, 2.3, 3.2)
8. Develop a robust system for testing the efficacy and stability of genetic containment mechanisms on a landscape scale (section 5)
9. Assess the effect and frequency of pollination of male sterile crop plants by cross-compatible wild relatives (section 3.2.3.3)
10. Conduct an assessment on the stability of genetic based containment mechanisms and the effect of natural methylation based silencing on their function during introgression (sections 3.2, 5)

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Additional important note

The reader of this report should be aware that all comments relating to the commercial aspects of research, and particularly any comments on the status of patents or patent applications, should not be taken as a definitive guide to these issues. Anyone requiring detailed information on commercial, intellectual property or legal issues should seek professional advice on these topics.

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Appendices

Appendix I

GM Field Trial Applications: online databases

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<http://www.ogtr.gov.au/index.htm>

<http://biotech.jrc.it/deliberate/gmo.asp>

<http://webdomino1.oecd.org/ehs/biotrack.nsf>

<http://www.inspection.gc.ca/english/plaveg/bio/triesse.shtml>

http://www.rki.de/gentec/geneng/gentec_e.htm

http://biosafety.abc.hu/biosafe_eng.html

<http://www.indiaresource.org/issues/agbiotech/2003/fieldsotrial.htm>

<http://www.s.affra.go.jp/docs/sentan/eguide/edevelp/htm>

<http://web2.senasica/sagarpa.gob.mx/portal/inocd/trser/doc4031>

<http://www.ermanz.govt.nz/no/index.asp>

<http://www.isb.vt.edu/cfdocs/fieldtests1.cfm>

Appendix II

Overseas Departments of Europe – online sources of agricultural data

CIA world fact book <http://www.cia.gov/cia/publications/factbook/>

<http://www.cr-guadeloupe.fr/economie>

<http://www.cr-guyane.fr>

<http://www.cr-martinique.fr>

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Appendix III

Patents and Patent Applications relating to GM crops and their containment

China

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Appendix IV

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
94-362-02r	U of Wisconsin	Alfalfa	Issued	04/12/95	Amylase from <i>Bacillus licheniformis</i> ; B-glucuronidase from <i>E. coli</i> ; Lignin peroxidase from <i>Phanerochaete chrysosporium</i>	Industrial enzyme	
97-052-02r	U of Wisconsin/Madison	Alfalfa	Issued	04/23/97	Cellulase; Inositol hexaphosphate phosphohydrolyase from <i>Aspergillus niger</i> ; Lignin peroxidase	Industrial enzyme	
93-088-02r	U of Wisconsin	Alfalfa	Issued	06/04/93	Alpha-amylase from <i>Bacillus licheniformis</i> ; Lignin peroxidase from <i>Phanerochaete chrysosporium</i>	Industrial enzyme	
92-183-01r	Noble Foundation	Alfalfa	Issued	10/10/92	Cholera toxin B from <i>Vibrio cholera</i>	Pharmaceutical proteins	
04-083-08n	Washington State U	Barley	Acknowledged	04/16/04	Lysozyme from Man; Phosphinothricin acetyl transferase*	Novel protein	1
00-334-01r	Washington State U	Barley	Issued	03/22/01	Amylase from Barley; Antithrombin from Man; Antitrypsin from Man; Green fluorescent protein*; Lactoferrin from Man; Lysozyme from Man; Phosphinothricin acetyl transferase*; Serum albumin from Man	Pharmaceutical proteins	3
00-054-01r	Applied Phytologics	Barley	Issued	04/18/00	CBI	Novel protein	3
04-104-01r	Ventria Bioscience	Barley	Issued	06/01/04	Hygromycin phosphotransferase*; Lactoferrin Man; Phosphinothricin acetyl transferase*	Value added protein for human consumption	0.05
00-228-02r	Applied Phytologics	Barley	Issued	10/10/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	
04-063-01r	Washington State U	Barley	Withdrawn	07/10/04		Pharmaceutical proteins	0.01
04-009-01r	ARS	Brassica juncea	Issued	03/30/04	ATP sulfurylase from <i>Arab. thaliana</i> ; Cystathionine synthase from <i>Arab. thaliana</i> ; Glutamylcysteine synthetase from <i>E. coli</i> ; Hygromycin phosphotransferase*; Methionine methyltransferase from <i>Arab. thaliana</i> ; NptII* SMT sulfurylase from <i>Astragalus bisulcatus</i> ; Selenocystine lyase from Mouse; Sodium/hydrogen ion exchanger from <i>Arab. thaliana</i>	Salt tolerance; Industrial enzyme	0.3
01-012-05n	ProdiGene	Corn	Acknowledged	03/12/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	1
01-012-06n	ProdiGene	Corn	Acknowledged	02/08/01	Phosphinothricin acetyl transferase*	Novel protein	1
01-229-05n	ProdiGene	Corn	Acknowledged	09/20/01	Phosphinothricin acetyl transferase*	Novel protein	
01-032-01n	ProdiGene	Corn	Acknowledged	02/12/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.5
01-045-06n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.15
94-279-06n	Agracetus	Corn	Acknowledged	11/02/94	CBI; Hygromycin phosphotransferase*	Antibody	0.3
00-228-03n	ProdiGene	Corn	Acknowledged	10/03/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.06
95-026-08n	Pioneer	Corn	Acknowledged	02/24/95	CBI; Phosphinothricin acetyl transferase*	Novel protein	24
95-026-06n	Pioneer	Corn	Acknowledged	02/24/95	CBI; Phosphinothricin acetyl transferase* from <i>Strep. hygroscopicus</i>	Novel protein	2
95-026-05n	Pioneer	Corn	Acknowledged	02/24/95	CBI; Phosphinothricin acetyl transferase*	Novel protein	2
95-026-04n	Pioneer	Corn	Acknowledged	03/01/95	CBI; Phosphinothricin acetyl transferase*	Novel protein	2
95-026-03n	Pioneer	Corn	Acknowledged	02/24/95	CBI; Phosphinothricin acetyl transferase*	Novel protein	22.5

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
00-224-03n	ProdiGene	Corn	Acknowledged	09/12/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.33
95-256-02n	Agracetus	Corn	Acknowledged	10/18/95	CBI; Hygromycin phosphotransferase* from E. coli	Antibody	2
00-228-05n	ProdiGene	Corn	Acknowledged	10/03/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.06
00-228-06n	ProdiGene	Corn	Acknowledged	09/12/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.07
00-221-03n	ProdiGene	Corn	Acknowledged	10/17/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	1
95-093-18n	Agracetus	Corn	Acknowledged	05/05/95	CBI; Hygromycin phosphotransferase* from E. coli	Antibody	1
95-093-19n	Agracetus	Corn	Acknowledged	05/10/95	CBI; Hygromycin phosphotransferase* from E. coli	Antibody	3
00-228-04n	ProdiGene	Corn	Acknowledged	09/01/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.25
02-081-10n	ProdiGene	Corn	Acknowledged	04/18/02	CBI; Phosphinothricin acetyl transferase*	Novel protein	
01-045-07n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.75
01-193-05n	ProdiGene	Corn	Acknowledged	08/09/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	
01-194-01n	ProdiGene	Corn	Acknowledged	08/22/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	
02-113-09n	ProdiGene	Corn	Acknowledged	05/22/02	Laccase from Turkey tails; Phosphinothricin acetyl transferase*	Industrial enzyme	5
01-194-02n	ProdiGene	Corn	Acknowledged	08/22/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	
02-081-13n	ProdiGene	Corn	Acknowledged	04/18/02	Brazzein from Pentadiplandra brazzeana; Phosphinothricin acetyl transferase*	Novel protein	
02-081-11n	ProdiGene	Corn	Acknowledged	04/29/02	Phosphinothricin acetyl transferase*	Novel protein	
02-081-08n	ProdiGene	Corn	Acknowledged	04/18/02	CBI; Phosphinothricin acetyl transferase*	Novel protein	
01-194-03n	ProdiGene	Corn	Acknowledged	08/24/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	
01-324-01n	ProdiGene	Corn	Acknowledged	01/09/02	Phosphinothricin acetyl transferase*	Novel protein	
02-081-12n	ProdiGene	Corn	Acknowledged	04/18/02	Laccase from Turkey tails; Phosphinothricin acetyl transferase*	Industrial enzyme	
95-335-02n	Pioneer	Corn	Acknowledged	12/21/95	CBI; Phosphinothricin acetyl transferase* from Strep. hygroscopicus	Novel protein	6
95-340-01n	Agracetus	Corn	Acknowledged	01/04/96	CBI; Hygromycin phosphotransferase* from E. coli	Antibody	1
01-045-08n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.36
01-045-09n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.54
01-045-10n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.06
01-190-02n	ProdiGene	Corn	Acknowledged	08/09/01	Laccase from Turkey tails; Phosphinothricin acetyl transferase*	Industrial enzyme	
01-045-12n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.12

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
01-150-01n	U of Hawaii	Corn	Acknowledged	06/25/01	Phosphinothricin acetyl transferase*; Polyhydroxybutyrate synthase from <i>Alcaligenes eutrophus</i> ; Aceto acetyl-CoA reductase from <i>Alcaligenes eutrophus</i> ; 3-ketothiolase from <i>Alcaligenes eutrophus</i>	Polymer	1
01-045-11n	ProdiGene	Corn	Acknowledged	03/16/01	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.12
99-055-10n	ProdiGene	Corn	Acknowledged	04/01/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.2
99-055-09n	ProdiGene	Corn	Acknowledged	04/01/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.2
00-060-06n	ProdiGene	Corn	Acknowledged	04/03/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	1.46
99-055-12n	ProdiGene	Corn	Acknowledged	04/01/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	4
98-356-05n	ProdiGene	Corn	Acknowledged	01/25/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.1
99-055-13n	ProdiGene	Corn	Acknowledged	04/01/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	2
98-273-11n	ProdiGene	Corn	Acknowledged	11/02/98	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.35
98-273-10n	ProdiGene	Corn	Acknowledged	11/02/98	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.35
99-278-01n	ProdiGene	Corn	Acknowledged	11/05/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.25
95-284-08n	Pioneer	Corn	Acknowledged	10/19/95	CBI; Phosphinothricin acetyl transferase*	Novel protein	6
99-271-05n	ProdiGene	Corn	Acknowledged	10/25/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.28
99-340-09n	ProdiGene	Corn	Acknowledged	01/04/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	12.2
99-340-08n	ProdiGene	Corn	Acknowledged	01/04/00	CBI; Phosphinothricin acetyl transferase*	Antibody	0.13
99-302-13n	ProdiGene	Corn	Acknowledged	11/23/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	20
99-294-04n	Pioneer	Corn	Acknowledged	11/08/99	CBI; CBI from Corn; Phosphinothricin acetyl transferase* from <i>Strep. hygroscopicus</i>	Visual marker; Phosphinothricin tolerant; Novel protein	
99-294-03n	Pioneer	Corn	Acknowledged	11/15/99	CBI; CBI from Corn; Phosphinothricin acetyl transferase* from <i>Strep. hygroscopicus</i>	Visual marker; Phosphinothricin tolerant; Novel protein	10
99-274-01n	ProdiGene	Corn	Acknowledged	11/01/99	CBI	Novel protein	0.35
99-055-11n	ProdiGene	Corn	Acknowledged	04/05/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	12
99-271-04n	ProdiGene	Corn	Acknowledged	10/25/99	CBI; Phosphinothricin acetyl transferase*	Antibody	0.33
99-271-03n	ProdiGene	Corn	Acknowledged	10/25/99	CBI	Novel protein	0.013
99-271-02n	ProdiGene	Corn	Acknowledged	10/25/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.16
00-049-13n	ProdiGene	Corn	Acknowledged	03/06/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	2
99-145-03n	ProdiGene	Corn	Acknowledged	06/02/99	CBI	Novel protein	15
99-106-11n	Iowa State U	Corn	Acknowledged	05/14/99	Phosphinothricin acetyl transferase*; Cecropin	Novel protein	
00-049-14n	ProdiGene	Corn	Acknowledged	03/06/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	25

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
99-055-15n	ProdiGene	Corn	Acknowledged	03/24/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.6
99-055-14n	ProdiGene	Corn	Acknowledged	04/01/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.05
96-317-03n	Agracetus	Corn	Acknowledged	12/06/96	CBI; Hygromycin phosphotransferase*	Antibody	3
00-109-07n	ProdiGene	Corn	Acknowledged	05/30/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	10
00-122-05n	ProdiGene	Corn	Acknowledged	06/13/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.15
97-098-07n	ProdiGene	Corn	Acknowledged	05/01/97	CBI; Phosphinothricin acetyl transferase*	Novel protein	5
96-094-08n	Agracetus	Corn	Acknowledged	04/23/96	CBI; Hygromycin phosphotransferase* from E. coli	Antibody	1
96-094-07n	Agracetus	Corn	Acknowledged	04/30/96	CBI; Hygromycin phosphotransferase*	Antibody	14
96-079-15n	Pioneer	Corn	Acknowledged	04/11/96	CBI; Phosphinothricin acetyl transferase* from Strep. hygroscopicus	Novel protein	3
96-079-14n	Pioneer	Corn	Acknowledged	03/26/96	CBI; Phosphinothricin acetyl transferase* from Strep. hygroscopicus	Novel protein	3
96-079-08n	Pioneer	Corn	Acknowledged	04/03/96	CBI; Phosphinothricin acetyl transferase* from Strep. hygroscopicus	Novel protein	3
00-192-08n	ProdiGene	Corn	Acknowledged	08/03/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	13
00-067-11n	ProdiGene	Corn	Acknowledged	04/18/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.1
98-085-42n	ProdiGene	Corn	Acknowledged	04/27/98	CBI; Phosphinothricin acetyl transferase*	Novel protein	4
98-068-12n	Monsanto	Corn	Acknowledged	04/10/98	CBI	Antibody	6
98-068-11n	Monsanto	Corn	Acknowledged	04/17/98	CBI	Antibody	204
96-317-02n	Agracetus	Corn	Acknowledged	12/06/96	CBI; Hygromycin phosphotransferase*	Antibody	3
00-067-10n	ProdiGene	Corn	Acknowledged	04/18/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	21.2
97-148-03n	Agracetus	Corn	Acknowledged	06/09/97	CBI; Hygromycin phosphotransferase*	Antibody	5
00-067-09n	ProdiGene	Corn	Acknowledged	04/18/00	CBI; Phosphinothricin acetyl transferase*	Novel protein	4.12
97-113-03n	Agracetus	Corn	Acknowledged	05/15/97	CBI; Hygromycin phosphotransferase*	Antibody	5.25
97-164-02n	Agracetus	Corn	Acknowledged	07/01/97	CBI; Hygromycin phosphotransferase*	Antibody	1
97-253-03n	ProdiGene	Corn	Acknowledged	10/09/97	CBI; Phosphinothricin acetyl transferase*	Novel protein	0.2
02-081-09n	ProdiGene	Corn	Denied	03/22/02		Novel protein	
00-060-04n	ProdiGene	Corn	Denied	03/07/00		Antibody	0.16
97-106-20n	Limagrain	Corn	Denied	04/18/97		Pharmaceutical proteins	
01-257-01r	Monsanto	Corn	Issued	01/11/02	CBI	Pharmaceutical proteins	20.8
98-274-02r	Monsanto	Corn	Issued	02/03/99	CBI	Pharmaceutical proteins	10
98-274-01r	Monsanto	Corn	Issued	02/04/99	CBI	Pharmaceutical proteins	1
99-343-01r	ProdiGene	Corn	Issued	02/22/00	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
99-034-03r	ProdiGene	Corn	Issued	03/03/99		Pharmaceutical proteins	

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
99-034-01r	ProdiGene	Corn	Issued	03/03/99	CBI; Phosphinothricin acetyl transferase* from Strep. viridochromogenes	Novel protein	
99-034-02r	ProdiGene	Corn	Issued	03/04/99	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
00-363-01r	Monsanto	Corn	Issued	03/21/01	C transcriptional activator	Pharmaceutical proteins	32
00-363-02r	Monsanto	Corn	Issued	03/21/01	C transcriptional activator	Pharmaceutical proteins	33.4
00-021-04r	Pioneer	Corn	Issued	03/30/00	Amino polyol amine oxidase; Aspartokinase from E. coli; B-glucuronidase*; CBI from Rapeseed; CBI from Soybean; CBI from Potato; CryIA(b) from Btk; DNA adenine methylase; from E. coli; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Esterase; Glycogenin from Corn; Glycogenin antisense from Corn; Luciferase*; Lysine ketoglutarate reductase from Corn; NptII*; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. Viridochromogenes; Saccharopine dehydrogenase from Corn; Starch branching enzyme II from Corn; Starch synthase from Corn; Storage protein from Corn	Altered maturing; Yield increased; Ear mold resistant; Smut resistant; Cyanamide tolerant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Novel protein; Animal feed quality; Fumonisin degradation; Grain processing improved	
00-021-03r	Pioneer	Corn	Issued	03/31/00	Amino polyol amine oxidase from Exophiala spinifera; Aspartokinase from E. coli; B-glucuronidase*; CBI; CBI from Potato; CBI from Soybean; CBI from Rapeseed; CBI from Pentataclethra macrebola; CryIA(b) from Btk; DNA adenine methylase from E. coli; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Esterase from Exophiala spinifera; Glycogenin from Corn; Glycogenin antisense from Corn; Luciferase*; Lysine ketoglutarate reductase from Corn; NptII*; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase ; Strep. viridochromogenes; Saccharopine dehydrogenase from Corn; Starch branching enzyme II from Corn; Starch synthase from Corn; Storage protein from Corn	Altered maturing; Yield increased; Ear mold resistant; Smut resistant; Cyanamide tolerance; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Novel protein; Animal feed quality; Fumonisin degradation; Grain processing improved	
02-023-01r	Pioneer	Corn	Issued	04/10/02	Aspartokinase from E. coli; CBI; Citrate lyase from Corn; Cystathionine synthase from Corn; Dehydroascorbate reductase from Corn; Dihydrodipicolinate synthase from Corn; Dihydrodipicolinate synthetase from Corynebacterium glutamicum; Lysine ketoglutarate reductase from Corn; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygrosopicus; Saccharopine dehydrogenase*; Starch synthase from Corn; Storage protein from Corn	Altered maturing; Fertility; Increased stalk strength; Ear mold resistant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Visual marker; Increased transformation frequency; Industrial enzyme; Novel protein; Animal feed quality; Fumonisin degradation	508
01-022-03r	Pioneer	Corn	Issued	04/13/01	Adenine methylase from E. coli; Amino polyol amine oxidase from Exophiala spinifera; B-glucuronidase*; CBI*; CBI frin Arabidopsis thaliana; CBI; CBI from Corn; CBI from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Esterase; Esterase from Exophiala spinifera; Flavin amine oxidase from Exophiala spinifera; Lysine ketoglutarate reductase from Corn; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. viridochromogenes; Phosphinothricin acetyl transferase from Strep. hygrosopicus; Saccharopine dehydrogenase from Corn; Storage protein from Corn	Altered maturing; Fertility; Fumonisin degradation; Increased stalk strength; Yield increased; Ear mold resistant; Phosphinothricin tolerant; Lepidopteran resistant; Novel protein; Animal feed quality; Grain processing improved	

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
01-022-04r	Pioneer	Corn	Issued	04/13/01	Adenine methylase from E. coli; Aspartokinase from Corn; CBI*; CBI; CBI from Soybean; CBI from Bt; CBI from Corn; CBI from E. coli; CBI - from Potato; CBI from Pentaclethra macrobola; CBI from Arabidopsis thaliana; Cry1F from Bt azawai; Dihydrodipicolinate synthase from Corn; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Esterase from ATTC 55552; Esterase from Exophila spinifera; Glycogenin from Corn; Lysine ketoglutarate reductase from Corn; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. viridochromogenes; Phosphinothricin acetyl transferase from Strep. hygroscopicus; Saccharopine dehydrogenase from Corn; Saccharopine dehydrogenase from Yarrowia lipolytica; Starch branching enzyme II antisense from corn; Starch synthase from corn; Storage protein from Corn	Yield increased; Ear mold resistant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Novel protein; Visual marker; Animal feed quality; Grain processing improved	
99-050-01r	Agracetus	Corn	Issued	04/14/99	CBI	Pharmaceutical proteins	24
01-016-01r	ProdiGene	Corn	Issued	04/17/01	CBI; NptII*	Pharmaceutical proteins	10
01-016-02r	ProdiGene	Corn	Issued	04/19/01	CBI; NptII*	Pharmaceutical proteins	0.7
01-016-03r	ProdiGene	Corn	Issued	04/19/01	CBI; NptII*	Pharmaceutical proteins	0.25
04-020-04r	Pioneer	Corn	Issued	04/20/04	Amino polyol amine oxidase from Exophila spinifera; B-glucuronidase*; CBI from Arab. thaliana; CBI from Rye; CBI from Rice; CBI from Pea; CBI from Corn; CBI; CBI from Barley; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Hygromycin phosphotransferase*; Luciferase*; Lysine ketoglutarate reductase from Corn; NptII*; Phosphinothricin acetyl transferase from Strep. hygroscopicus; Storage protein from Corn	Altered maturing; Fertility; Increased stalk strength; Yield increased; Ear mold resistant; Phosphinothricin tolerant; Increased transformation frequency; Novel protein; Animal feed quality; Fumonisin degradation; Grain processing improved	3200
04-020-03r	Pioneer	Corn	Issued	04/20/04	Amino polyol amine oxidase from Exophila spinifera; B-glucuronidase*; C1 regulatory gene from Corn; CBI from Arab. thaliana; CBI from Corn; CBI from Pea; CBI from Rice; CBI from Rye; CBI; CBI from Barley; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Hygromycin phosphotransferase*; Luciferase*; Lysine ketoglutarate reductase from Corn; NptII*; Phosphinothricin acetyl transferase from Strep. hygroscopicus; Storage protein from Corn	Altered maturing; Fertility; Increased stalk strength; Yield increased; Ear mold resistant; Phosphinothricin tolerant; Increased transformation frequency; Novel protein produced; Animal feed quality improved; Fumonisin degradation; Grain processing improved	7475
01-057-01r	Horan Bros. Agri. Enterprises	Corn	Issued	04/25/01	CBI; NptII*	Pharmaceutical proteins	33.2
00-067-02r	ProdiGene	Corn	Issued	04/26/00	CBI	Pharmaceutical proteins	
04-040-01r	ProdiGene	Corn	Issued	04/28/04	Aprotinin from Cow; Brazzein from Pentadiplandra brazzeana; CBI; CBI from Man; Epitope from CBI; Epitope from TGEV; Glycoprotein gp120 from Human immunodeficiency virus; Phosphinothricin acetyl transferase*; Trypsin from Cow	Novel protein	
00-067-01r	ProdiGene	Corn	Issued	05/01/00	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	40
01-023-03r	ProdiGene	Corn	Issued	05/08/01	Aprotinin from Bos taurus; CBI; Enterotoxin subunit B from E. coli; NptII*; Phosphinothricin acetyl transferase*; Surface antigen from Hepatitis virus B; Surface antigen from TGEV; gp120 (glycoprotein 120) - from Simian immunodeficiency virus	Pharmaceutical proteins	53.5
01-023-04r	ProdiGene	Corn	Issued	05/08/01	CBI; NptII*	Pharmaceutical proteins	0.25

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
05-024-03r	Pioneer	Corn	Issued	05/12/05	Acetolactate synthase* from Soybean; Adenine methylase from E. coli; B-glucuronidase* from E. coli; C1 regulatory gene* from Corn; CBI from Secale cereale; CBI from Corn; CBI from Rice; CBI; CBI*; CBI* from Corn; CBI from Arabidopsis thaliana; CBI from Barley; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Hygromycin phosphotransferase* from E. coli; Luciferase* from Photinus pyralis; Lysine ketoglutarate reductase from Corn; Phosphinothricin acetyl transferase from Strep. viridochromogenes; Phosphinothricin acetyltransferase from Strep. hygroscopicus; Storage protein from Barley	Altered plant development; Altered stalk attributes; Fertility; Maturity; Reduced plant growth; Yield increased; Yield stability; Glyphosate tolerant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Selectable marker; Visual marker; Increased transformation frequency; Novel protein; Amino acid composition; Starch content; Digestibility improved; Fatty acid levels; Feed properties; Fiber quality; Fumonisin degradation; Fumonisin mycotoxin levels; Grain processing improved; Lipid profile; Nutritional quality; Oil profile; Phytate reduced; Protein lysine level increased; Protein quality; Starch level increased; Starch metabolism	10285
02-108-01r	Meristem Therapeutics	Corn	Issued	05/21/02	Antibody (common cold) from Mouse; Antibody (tooth decay) from Mouse; NptII*	Pharmaceutical proteins	5
05-024-04r	Pioneer	Corn	Issued	05/25/05	Acetolactate synthase*; Adenine methylase from E. coli; Amino polyol amine oxidase from Exophila spinifera; B-glucuronidase*; C1 regulatory gene*; CBI from Arab. thaliana; CBI*; CBI from Barley; CBI from Corn; CBI; CBI from Rice; CBI from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Hemagglutinin epitope*; Hygromycin phosphotransferase*; Luciferase*; Lysine ketoglutarate reductase from Corn; Phosphinothricin acetyltransferase from Strep. viridochromogenes; Phosphinothricin acetyltransferase*; Phosphinothricin acetyltransferase from Strep. hygroscopicus	Altered plant developmen; Stalk attributes; Fertility; Maturity; Reduced plant growth; Yield enhancement; Yield stability; Glyphosate tolerant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Selectable marker; Visual marker; Increased transformation frequency; Novel protein; Amino acid composition; Starch content; Digestibility improved; Fatty acid levels; Feed properties; Fiber quality; Fumonisin degradation; Grain processing improved; Lipid profile; Lysine level increased; Nutritional quality; Oil profile; Phytate reduced; Protein quality; Starch levels; Starch metabolism	2800
00-073-01r	ProdiGene	Corn	Issued	05/26/00	CBI*; Phosphinothricin acetyl transferase	Pharmaceutical proteins	2
04-131-01r	Iowa State U	Corn	Issued	06/01/04	Enterotoxin subunit B from E. coli; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	0.25
02-141-01r	Meristem Therapeutics	Corn	Issued	06/05/02	CBI*; CBI from Man; CBI	Pharmaceutical proteins	1

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
03-086-01r	Meristem Therapeutics	Corn	Issued	06/05/03	CBI*; CBI	Pharmaceutical proteins	
02-071-01r	Dow	Corn	Issued	06/07/02	CBI	Pharmaceutical proteins	
05-069-01r	Iowa State U	Corn	Issued	06/10/05	LT-B heat labile enterotoxin from E. coli; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	0.1
98-117-03r	Limagrain	Corn	Issued	06/10/98	Phosphinothricin acetyl transferase*; Serum albumin from Man	Pharmaceutical proteins	0.5
98-117-02r	Limagrain	Corn	Issued	06/12/98	Phosphinothricin acetyl transferase; Procollagen* from Man	Pharmaceutical proteins	0.2
98-117-04r	Limagrain	Corn	Issued	06/12/98	G glycoprotein	Pharmaceutical proteins	0.5
98-117-01r	Limagrain	Corn	Issued	06/12/98	Alpha-hemoglobin from Man; Beta-hemoglobin from Man; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	0.25
01-122-01r	Iowa State U	Corn	Issued	06/14/01	Enterotoxin subunit B from E. coli; NptII*	Pharmaceutical proteins	0.2
01-114-01r	ProdiGene	Corn	Issued	07/12/01	CBI; NptII*	Pharmaceutical proteins	1
00-122-01r	ProdiGene	Corn	Issued	07/14/00	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
00-195-01r	ProdiGene	Corn	Issued	08/23/00	CBI*; CBI; Phosphinothricin acetyl transferase*	Novel protein	0.5
01-190-01r	ProdiGene	Corn	Issued	10/05/01	Enterotoxin subunit B from E. coli; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
00-203-04r	ProdiGene	Corn	Issued	10/13/00		Pharmaceutical proteins	0.1
03-143-01r	Garst	Corn	Issued	10/14/03	Acetolactate synthase*; CBI from Man; CBI from Mouse	Pharmaceutical proteins	36
01-212-01r	Dow	Corn	Issued	10/23/01	CBI from Human; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	7.9
00-221-01r	ProdiGene	Corn	Issued	11/03/00	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
00-203-01r	Monsanto	Corn	Issued	11/03/00	CBI*; CBI	Pharmaceutical proteins	15
00-203-02r	Monsanto	Corn	Issued	11/03/00	CBI*; CBI	Pharmaceutical proteins	15
00-203-03r	Monsanto	Corn	Issued	11/03/00	CBI*; CBI	Pharmaceutical proteins	15
01-187-01r	ProdiGene	Corn	Issued	11/13/01	Aprotinin from Pig; CBI; Phosphinothricin acetyl transferase*; Surface antigen from Hepatitis virus B; Surface antigen from TGEV; gp120 (glycoprotein 120) from SIV	Pharmaceutical proteins	0.4
99-279-02r	ProdiGene	Corn	Issued	11/18/99	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
99-279-01r	ProdiGene	Corn	Issued	11/18/99	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	0.2
99-307-01r	ProdiGene	Corn	Issued	11/18/99	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
98-257-01r	ProdiGene	Corn	Issued	11/25/98	CBI; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	
00-207-01r	Monsanto	Corn	Issued	12/01/00	CBI*; CBI	Pharmaceutical proteins	15
99-307-02n	ProdiGene	Corn	Withdrawn	11/23/99	CBI; Phosphinothricin acetyl transferase*	Novel protein	20
98-068-13n	Monsanto	Corn	Withdrawn	05/05/98	CBI*; CBI	Antibody	12
04-121-02r	ProdiGene	Corn	Withdrawn	07/02/04	Brazzein from Pentadiplandra brazzeana; CBI; CBI from Man; Glycoprotein gp120 from Human immunodeficiency virus; Phosphinothricin acetyl transferase*	Value added protein	
04-121-01r	ProdiGene	Corn	Withdrawn	08/25/04	Aprotinin from Cow; Phosphinothricin acetyl transferase*	Value added protein	
04-114-02r	ProdiGene	Corn	Withdrawn	08/25/04	Phosphinothricin acetyl transferase*; Trypsin from Cow	Value added protein	
04-065-01r	Iowa State U	Corn	Withdrawn	09/07/04	Enterotoxin subunit B from E. coli; Phosphinothricin acetyl transferase*	Pharmaceutical proteins	0.25
97-273-11n	Monsanto	Rapeseed	Denied	10/02/97		Polymer	2
97-266-06n	Monsanto	Rapeseed	Denied	09/24/97		Polymer	1
96-061-02r	Monsanto	Rapeseed	Issued	04/16/96	CBI*; CBI	Polymer	
93-048-02r	Cargill	Rapeseed	Issued	05/04/93	CBI; NptII*	Industrial enzyme	

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
96-215-01r	Pioneer	Rapeseed	Issued	09/17/96	CBI; Phosphinothricin acetyl transferase* from Strep. Hygroscopicus	Pharmaceutical proteins	
97-283-01n	Monsanto	Rapeseed	Withdrawn	10/20/97	CBI; CBI*	Polymer	2
01-206-01r	Applied Phytologics	Rice	Issued		Antitrypsin from Human; CBI from Human; Dirigent protein from Forsythia intermedia; Hygromycin phosphotransferase*; Laccase from Forsythia intermedia; Lactoferrin from Human; Lysozyme from Human; Pinorensinol reductase from Forsythia intermedia; Pinorensinol-laricresinol reductase from Forsythia intermedia; Secoisolaricresinol dehydrogenase from Forsythia intermedia	Novel protein	
97-363-01r	Applied Phytologics	Rice	Issued	02/25/98	CBI; Hygromycin phosphotransferase*	Pharmaceutical proteins	
98-008-01r	Applied Phytologics	Rice	Issued	02/25/98	Antithrombin from Man; Antitrypsin from Man; Hygromycin phosphotransferase* from E. coli; Serum albumin from Man	Pharmaceutical proteins	
96-355-01r	Applied Phytologics	Rice	Issued	03/31/97	Aminoglycoside 3'-adenylyltransferase from Man; Antithrombin from Man; Hygromycin phosphotransferase*; Serum albumin from Man	Pharmaceutical proteins	
05-073-01r	Ventria Bioscience	Rice	Issued	04/06/05	Lactoferrin from Man; Lysozyme from Man; Serum albumin from Man	Value added protein	4.5
02-361-01r	Ventria Bioscience	Rice	Issued	04/14/03	Hygromycin phosphotransferase*; Lactoferrin from Man; Lysozyme from Man; Phosphinothricin acetyl transferase*; Seed storage protein antisense from Rice	Value added protein for human consumption	93
01-029-02r	Applied Phytologics	Rice	Issued	04/19/01	Antitrypsin from Man; CBI; CBI from Man; Lactoferrin from Man; Lysozyme from Man; NptII*	Pharmaceutical proteins; Gene expression; Novel protein; Storage protein	100
03-365-01r	Ventria Bioscience	Rice	Issued	05/13/04	Hygromycin phosphotransferase*; Lactoferrin from Man; Lysozyme from Man; Phosphinothricin acetyl transferase*	Value added protein for human consumption	1
00-069-01r	Applied Phytologics	Rice	Issued	05/15/00	CBI; Hygromycin phosphotransferase*; NptII*	Pharmaceutical proteins; Novel protein	7
05-117-01r	Ventria Bioscience	Rice	Issued	06/28/05	Lactoferrin from Man	Value added protein for human consumption	35
05-117-02r	Ventria Bioscience	Rice	Issued	06/28/05	Lysozyme from Man	Value added protein for human consumption	35
00-217-01r	Applied Phytologics	Rice	Issued	10/06/00		Novel protein	
99-272-01r	Applied Phytologics	Rice	Issued	10/28/99	CBI; Hygromycin phosphotransferase*	Pharmaceutical proteins	
99-272-02r	Applied Phytologics	Rice	Issued	10/28/99	CBI; Hygromycin phosphotransferase*	Pharmaceutical proteins	
04-309-01r	Ventria Bioscience	Rice	Withdrawn	06/20/05	Lysozyme from Man	Value added protein for human consumption	100
04-302-01r	Ventria Bioscience	Rice	Withdrawn	06/20/05	Lactoferrin from Man	Value added protein for human consumption	100
05-004-01r	Ventria Bioscience	Rice	Withdrawn	06/20/05	Lactoferrin from Man; Lysozyme from Man; Serum albumin from Man	Value added protein for human consumption	4.5
02-099-13n	Emlay and Associates	Safflower	Acknowledged	05/28/02	CBI; Phosphinothricin acetyl transferase*	Novel protein	1
03-071-01r	Emlay and Associates	Safflower	Issued	06/03/03	CBI from Cow; Cystatin proteinase from Arab. Thaliana; Growth hormone from Carp; Oleosin from Arab. Thaliana; Phosphinothricin acetyl transferase*	Industrial enzymes; Pharmaceutical proteins	11
96-113-05n	Agracetus	Soybean	Acknowledged	05/20/96	CBI; B-glucuronidase* from E. coli	Antibody	0.1

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
94-257-05n	Agracetus	Soybean	Acknowledged	10/04/94	CBI; NptII*	Antibody	5
95-093-17n	Agracetus	Soybean	Acknowledged	05/05/95	CBI; B-glucuronidase* from E. coli	Antibody	1
94-279-05n	Agracetus	Soybean	Acknowledged	11/02/94	CBI; B-glucuronidase*	Antibody	0.5
95-074-05n	Agracetus	Soybean	Acknowledged	04/17/95	CBI; B-glucuronidase* from E. coli	Antibody	0.1
95-074-06n	Agracetus	Soybean	Acknowledged	04/17/95	CBI; B-glucuronidase* from E. coli	Antibody	0.1
95-093-16n	Agracetus	Soybean	Acknowledged	05/05/95	CBI; B-glucuronidase* from E. coli	Antibody	1
95-101-04n	Agracetus	Soybean	Acknowledged	05/12/95	CBI; B-glucuronidase* from E. coli	Antibody	1
95-268-05n	Agracetus	Soybean	Acknowledged	10/26/95	CBI; B-glucuronidase* from E. coli	Antibody	1
96-086-04n	Monsanto	Soybean	Acknowledged	04/09/96	CBI; CBI*	Polymer	2
94-136-01n	Agracetus	Soybean	Acknowledged	05/24/94	CBI; B-glucuronidase*	Industrial enzyme	0.5
96-113-04n	Agracetus	Soybean	Acknowledged	05/01/96	CBI; B-glucuronidase* from E. coli	Antibody	0.3
95-019-04n	Agracetus	Soybean	Acknowledged	02/09/95	CBI; B-glucuronidase*	Industrial enzyme	0.1
96-172-02n	Agracetus	Soybean	Acknowledged	08/01/96	CBI; B-glucuronidase* from E. coli	Antibody	1
96-198-01n	Agracetus	Soybean	Acknowledged	07/22/96	CBI; B-glucuronidase* from E. coli	Industrial enzyme	0.1
96-198-03n	Agracetus	Soybean	Acknowledged	07/22/96	CBI; B-glucuronidase* from E. coli	Antibody; Novel protein	0.1
96-277-06n	Agracetus	Soybean	Acknowledged	10/16/96	CBI; B-glucuronidase* from E. coli	Industrial enzyme	0.2
99-074-02n	Monsanto	Soybean	Acknowledged	04/19/99	CBI; CBI*	Industrial enzyme	6
96-113-02n	Agracetus	Soybean	Acknowledged	05/20/96	CBI; B-glucuronidase* from E. coli	Industrial enzyme	0.3
93-321-01n	Agracetus	Soybean	Acknowledged	12/13/93	CBI; B-glucuronidase* from E. coli	Industrial enzyme	0.1
00-021-02r	Pioneer	Soybean	Issued	03/30/00	Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase from E. coli; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase*; CBI*; CBI from Corn; CBI from Soybean; CBI; Conglycinin from Soybean; Cystathionine beta-lyase from Soybean; Cystathionine synthase from Corn; Cystathionine synthase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from Soybean; Dihydrodipicolinate synthase from E. coli; Galactinol synthase from Soybean; Glycinin from Soybean; Hygromycin phosphotransferase*; Isoflavone synthase from Soybean; Luciferase*; Lysine ketoglutarate reductase from Soybean; NptII*; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase*; Saccharopine dehydrogenase from Soybean; Saccharopine dehydrogenase from Yarrowia lipolytica; Storage protein from Corn; Storage protein from Synthetic; UDP-glucose 4'epimerase from Soybean	Altered maturing; Yield increased; Ear mold resistant; Cyanamide tolerant; Phosphinothricin tolerant; Coleopteran resistant; Lepidopteran resistant; Novel protein; Animal feed quality; Fumonisin degradation; Grain processing improved	

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
02-023-05r	Pioneer	Soybean	Issued	04/10/02	Acetolactate synthase* from Arab. thaliana; Acetolactate synthase from Arab. thaliana; Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase* from E. coli; B-glucuronidase from Tobacco; CBI from Alfalfa; CBI from Arab. thaliana; CBI from Soybean; CBI; Conglycinin from Soybean; Cystathionine synthase from Corn; Cystathionine synthase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthetase from Corynebacterium glutamicum; Galactinol synthase from Soybean; Hygromycin phosphotransferase* from E. coli; Hygromycin phosphotransferase from E. coli; Lysine ketoglutarate reductase from Soybean; NptII* from E. coli; NptII from Tobacco; NptII from E. coli; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase* from Strep. viridochromogenes; Phosphinothricin acetyl transferase from Strep. viridochromogenes; Saccharopine dehydrogenase; Seed storage protein from Synthetic; Storage protein from Corn; Thioesterase from Rapeseed; Thioesterase from Soybean; UDP-glucose 4'epimerase from Soybean	Fungal susceptibility; Sclerotinia resistant; Increased transformation frequency; Novel protein; Animal feed quality; Yield increased	182
04-020-01r	Pioneer	Soybean	Issued	04/13/04	Acetolactate synthase*; Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase from E. coli; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase*; CBI*; CBI from Agro. tumefaciens; CBI from Arab. thaliana; CBI from Vernonia galamensis; CBI from Thraustochytrium aureum; CBI from Stigmatella aurantiaca; CBI from Soybean; CBI from Schizochytrium aggregatum; CBI from Saprolegnia diclina; CBI from Rice; CBI from Parthenium argentatum; CBI from Mortierella alpina; CBI from Isochrysis galabana; CBI from Euphorbia lagascae; CBI from Corn; CBI; CBI from Barley; CBI from Bacillus subtilis; CBI from Bacillus sp.; CBI from Bacillus amyloliquefaciens; CBI from Alfalfa; Conglycinin from Soybean; Cystathionine beta-lyase from Soybean; Cystathionine synthase from Soybean; Delta-12 desaturase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from Soybean; Galactinol synthase from Soybean; Glycinin from Soybean; Hygromycin phosphotransferase*; Luciferase*; Lysine ketoglutarate reductase from Soybean; NptII*; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygrosopicus; Saccharopine dehydrogenase from Yarrowia lipolytica; Storage protein from Corn; UDP-glucose 4'epimerase from Soybean	Longer stems; Shorter stems; Yield increased; Sclerotinia resistant; Glyphosate tolerant; Phosphinothricin tolerant; Fungal susceptibility; Novel protein; Transformation frequency increased; Animal feed quality	410
03-022-03r	Pioneer	Soybean	Issued	04/15/03	10 kDa protein from Corn; Acetolactate synthase*; Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase*; CBI*; CBI from Arab. thaliana; CBI from Alfalfa; CBI from Bacillus amyloliquefaciens; CBI from Corn; CBI from Isochrysis galabana; CBI from Parthenium argentatum; CBI from Wheat; CBI from Vernonia galamensis; CBI from Thraustochytrium aureum; CBI from Sunflower; CBI from Soybean; CBI from Schizochytrium aggregatum; CBI from Saprolegnia diclina; CBI from Rice; CBI from Mortierella alpina; CBI from Euphorbia lagascae; CBI; Conglycinin from Soybean; Cystathionine beta-lyase from Soybean; Cystathionine synthase from Corn; Cystathionine synthase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from Soybean; Dihydrodipicolinate synthase from E. coli; Galactinol synthase from Soybean; Hygromycin phosphotransferase*; Lysine ketoglutarate reductase from Soybean; NptII*; Omega 3 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygrosopicus; Storage protein from Corn; UDP-glucose 4'epimerase from Soybean	Sclerotinia resistant; Glyphosate tolerant; Phosphinothricin tolerant; Fungal susceptibility; Increased transformation frequency; Novel protein produced; Animal feed quality; Yield increased; Fusarium resistant	520
04-020-02r	Pioneer	Soybean	Issued	04/15/04	Acetolactate synthase*; Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase from E. coli; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase*; CBI*; CBI from Alfalfa; CBI from Bacillus sp.; CBI from Barley; CBI from Bacillus amyloliquefaciens; CBI from Arab. thaliana; CBI from Corn; CBI from Isochrysis galabana; CBI from Vernonia galamensis; CBI from Thraustochytrium aureum; CBI from Stigmatella aurantiaca; CBI from Soybean; CBI from Schizochytrium aggregatum; CBI from Saprolegnia diclina; CBI from Rice; CBI from Parthenium argentatum; CBI from Mortierella alpina; CBI from Euphorbia lagascae; Conglycinin from Soybean; Cystathionine beta-lyase from Soybean; Cystathionine synthase from Soybean; Delta-12 saturase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from Soybean; Galactinol synthase from Soybean; Glycinin from Soybean; Hygromycin phosphotransferase*; Luciferase*; Lysine ketoglutarate reductase from Soybean; NptII*; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygrosopicus; Saccharopine dehydrogenase from Yarrowia lipolytica; Storage protein from Corn; UDP-glucose 4'epimerase from Soybean	Longer stems; Shorter stems; Yield increased; Sclerotinia resistant; Glyphosate tolerant; Phosphinothricin tolerant; Fungal susceptibility; Novel protein; Transformation frequency increased; Animal feed quality	100

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
03-022-04r	Pioneer	Soybean	Issued	05/01/03	10 kDa protein from Corn; Acetolactate synthase*; Acyl-ACP thioesterase from Rapeseed; Acyl-ACP thioesterase from Soybean; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase*; CBI*; CBI from Alfalfa; CBI - from Bacillus amyloliquefaciens; CBI from Corn; CBI from Isochrysis galabana; CBI from Vernonia galamensis; CBI from Thraustochytrium aureum; CBI from Sunflower; CBI from Soybean; CBI from Schizochytrium aggregatum; CBI from Saprolegnia diclina; CBI from Rice; CBI from Parthenium argentatum; CBI from Mortierella alpina; CBI from Euphorbia lagascae; CBI; CBI from Arab. thaliana; Conglycinin from Wheat; Cystathionine beta-lyase from Soybean; Cystathionine synthase from Corn; Cystathionine synthase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from Soybean; Dihydrodipicolinate synthase from E. coli; Galactinol synthase from Soybean; Hygromycin phosphotransferase*; Lysine ketoglutarate reductase from Soybean; NptII*; Omega 3 desaturase from Soybean; Omega 6 desaturase antisense from Soybean; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygroscopicus; Storage protein from Corn; UDP-glucose 4'epimerase from Soybean	Yield increased; Sclerotinia resistant; Glyphosate tolerant; Phosphinothricin tolerant; Fungal susceptibility; Increased transformation frequency; Novel protein; Animal feed quality; Nutritional quality improved; Oil quality	80
05-024-01r	Pioneer	Soybean	Issued	05/12/05	Acetolactate synthase*; Acyl-ACP thioesterase from Soybean; Acyl-ACP thioesterase from Rapeseed; Aspartokinase from E. coli; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase - Donor: E. coli; B-glucuronidase*; C1 regulatory gene from Corn; CBI from Alfalfa; CBI from Schizochytrium aggregatum; CBI from Parthenium argentatum; CBI from Euphorbia lagascae; CBI; CBI from Arab. thaliana; CBI - from Barley; CBI; CBI from Corn; CBI from Mortierella alpina; CBI from Rice; CBI from Soybean; CBI from Bacillus amyloliquefaciens; CBI from Vernonia galamensis; CBI from Sunflower; Conglycinin from Soybean; Cystathionine beta lyase from Soybean; Cystathionine synthase from Soybean; Cystathionine synthase from Corn; Delta-12 saturase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from E. coli; Galactinol synthase from Soybean; Glycinin from Soybean; Hygromycin phosphotransferase*; Luciferase from Jellyfish; Lysine ketoglutarate reductase from Soybean; Lysine- and Methionine-rich protein from Synthetic; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Phosphinothricin acetyl transferase*; Phosphinothricin acetyl transferase from Strep. hygroscopicus; Saccharopine dehydrogenase from Yarrowia lipolytica	Stem attributes; Yield increased; Yield stability; Fungal susceptibility; Mold resistance; Sclerotinia resistant; Phosphinothricin tolerant; Aphid resistant; Visual marker; Increased transformation frequency; Novel protein; Amino acid composition; Carbohydrate metabolism; Cell wall; Fatty acid levels; Feed properties; Flavinoid levels; Flavinoid level decreased; Food quality; Lipid profile; Lysine level increased; Nutritional quality; Oil quality	915
05-024-02r	Pioneer	Soybean	Issued	05/25/05	ACP acyl ACP thioesterase from Rapeseed; Acetolactate synthase*; Acyl-ACP thioesterase from Soybean; Aspartokinase from E. coli; Aspartokinase II-homoserine dehydrogenase from E. coli; B-glucuronidase from E. coli; B-glucuronidase*; C1 regulatory gene from Corn; CBI from Euphorbia lagascae; CBI; CBI from Bacillus amyloliquefaciens; CBI*; CBI from Mortierella alpina; CBI from Parthenium argentatum; CBI from Rice; CBI from Schizochytrium aggregatum; CBI from Soybean; CBI from Sunflower; CBI from Vernonia galamensis; CBI from Alfalfa; CBI from Arab. thaliana; CBI from Barley; CBI from Corn; Conglycinin from Soybean; Cystathionine beta lyase from Soybean; Cystathionine synthase from Soybean; Cystathionine synthase from Corn; Delta-12 saturase from Soybean; Delta-9 desaturase from Soybean; Dihydrodipicolinate synthase from Soybean; Dihydrodipicolinate synthase from Corynebacterium glutamicum; Dihydrodipicolinate synthase from E. coli; Galactinol synthase from Soybean; Glycinin from Soybean; Hygromycin phosphotransferase*; Luciferase from Photinus pyralis; Lysine ketoglutarate reductase from Soybean; Lysine- and Methionine-rich protein from Synthetic; Omega 3 desaturase from Soybean; Omega 6 desaturase from Soybean; Phosphinothricin acetyl transferase from Arab. thaliana; Saccharopine dehydrogenase from Yarrowia lipolytica; Storage protein from Corn; UDP-glucose 4'epimerase from Soybean	Stem attributes; Yield increased; Yield stability; Fungal susceptibility; Mold resistance; Sclerotinia resistant; Phosphinothricin tolerant; Aphid resistant; Visual marker; Increased transformation frequency; Novel protein; Amino acid composition; Carbohydrate metabolism; Cell wall; Fatty acid levels; Feed properties; Flavinoid levels; Flavinoid level decreased; Lipid profile; Lysine level increased; Nutritional quality; Oil quality; Protein quality	125
99-147-02n	Monsanto	Soybean	Withdrawn	08/25/99	CBI; CBI*	Industrial enzyme	10
01-306-01r	Hawaii Agriculture Research Ce	Sugarcane	Issued	01/11/02	NptII*; CBI from Man	Pharmaceutical proteins	0.5
96-184-04n	Biosource	Tobacco	Acknowledged	07/18/96	CBI	Novel protein	20
99-041-02r	CropTech	Tobacco	Issued	03/08/99	CBI; NptII*	Pharmaceutical proteins	0.4

Field trial applications of GM crops expressing pharmaceutical and industrial products in the US *continued*

Permit	Institution	Organism	Status	Issued/ Effective	Gene(s)	Phenotype(s)/Product	Acreage
99-041-01r	CropTech	Tobacco	Issued	03/08/99	CBI; NptII*	Pharmaceutical proteins	0.4
00-070-01r	CropTech	Tobacco	Issued	04/19/00	CBI from Man; NptII*	Pharmaceutical proteins	
00-070-02r	CropTech	Tobacco	Issued	04/19/00	CBI from Man; NptII*	Pharmaceutical proteins	
04-044-01r	Planet Biotechnology	Tobacco	Issued	04/28/04	Antibody from Mouse; Antibody from Oryctolagus cuniculus	Pharmaceutical proteins	2
02-080-01r	CropTech	Tobacco	Issued	05/07/02	CBI from Man; NptII*	Pharmaceutical proteins	0.5
01-074-01r	CropTech	Tobacco	Issued	05/11/01	CBI	Pharmaceutical proteins	
01-074-02r	CropTech	Tobacco	Issued	05/11/01	CBI	Pharmaceutical proteins	
03-063-01r	Chlorogen, Inc.	Tobacco	Issued	05/15/03	Amino glycoside adenylyl transferase*; Serum albumin from Man	Pharmaceutical proteins	0.1
04-355-01r	Chlorogen, Inc.	Tobacco	Issued	05/19/05	Amino glycoside adenylyl transferase*; CBI from Man; CBI from Cow	Pharmaceutical proteins	12.5
04-114-01r	Chlorogen, Inc.	Tobacco	Issued	05/25/04	Albumin from Man; Aminoglycoside 3'-adenylyltransferase*; Insulin-like growth factor from Man; Interferon from Man; Mullerian inhibiting substance from Man	Pharmaceutical proteins	1
05-061-01r	U of Kentucky	Tobacco	Issued	06/07/05	NptII*; Phenylalanine ammonia lyase from Arab. thaliana	Pharmaceutical proteins	0.25
05-053-01r	Planet Biotechnology	Tobacco	Issued	06/08/05	Antibody (common cold) from CBI; Antibody (tooth decay) from Mouse; Antibody (tooth decay) from Oryctolagus cuniculus; NptII*	Pharmaceutical proteins	2
05-087-01r	Planet Biotechnology	Tobacco	Issued	07/07/05	Antibody (tooth decay) from Mouse	Pharmaceutical proteins	10
04-077-01r	Chlorogen, Inc.	Tobacco	Withdrawn	07/27/04	Aminoglycoside acetyltransferase*; Cholera toxin B from Vibrio cholera; Insulin-like growth factor from Man; Interferon from Man; Mullerian inhibiting substance from Man; Protective antigen from Bacillus anthracis; Protective antigen from Yersinia pestis; Serum albumin from Man	Pharmaceutical proteins	3
97-301-01r	ProdiGene	Tomato	Issued	01/29/98	NptII*	Pharmaceutical proteins	1
00-059-01r	Applied Phytologics	Wheat	Issued	05/04/00	CBI; Hygromycin phosphotransferase*	Novel protein	3
00-228-01r	Applied Phytologics	Wheat	Issued	10/10/00	CBI; Hygromycin phosphotransferase*	Novel protein	

Appendix V

Deregulated GM crops in the US

Applicant Documents						APHIS Documents			
Petition	Extension of Petition Number ***	Institution	Regulated article	Transgenic Phenotype	Transformation Event or Line	Preliminary EA ****	FR Notice	Risk Asses.	Final EA & Determination
92-196-01p		Calgene	Tomato	Fruit ripening altered	FLAVR SAVR	92-196-01p_ea	92-196-01p_fr		92-196-01p_com
92-204-01p		Upjohn	Squash	WMV2 & ZYMV resistant	ZW-20	92-204-01p_ea	92-204-01p_fr		92-204-01p_com
93-196-01p		Calgene	Cotton	Bromoxynil tolerant	BXN	93-196-01p	93-196-01p_fr		93-196-01p_com
93-258-01p		Monsanto	Soybean	Glyphosate tolerant	40-3-2	93-258-01p	93-258-01p		93-258-01p_com
94-090-01p		Calgene	Rapeseed	Oil profile altered	pCGN3828-212/86- 18 & 23	94-090-01p	94-090-01p		94-090-01p_com
94-227-01p	92-196-01p	Calgene	Tomato	Fruit ripening altered	Line N73 1436-111	94-227-01p	94-227-01p_fr		94-227-01p_com
94-228-01p		DNA Plant Tech	Tomato	Fruit ripening altered	1345-4	94-228-01p_ea	94-228-01p_fr		94-228-01p_com
94-230-01p	92-196-01p	Calgene	Tomato	Fruit ripening altered	9 additional FLAVRSAVR lines	94-230-01p	94-230-01p		94-230-01p_com
94-257-01p		Monsanto	Potato	Coleopteran resistant	BT6, BT10, BT12, BT16, BT17, BT18, BT23	94-257-01p_ea	94-257-01p_fr		94-257-01p_com
94-290-01p		Zeneca & Petoseed	Tomato	Fruit polygalacturonase level decreased	B, Da, F	94-290-01p	94-290-01p		94-290-01p_com
94-308-01p		Monsanto	Cotton	Lepidopteran resistant	531, 757, 1076	94-308-01p	94-308-01p		94-308-01p_com
94-319-01p		Ciba Seeds	Corn	Lepidopteran resistant	Event 176	94-319-01p	94-319-01p		94-319-01p_com
94-357-01p		AgrEvo	Corn	Phosphinothricin tolerant	T14, T25	94-357-01p	94-357-01p		94-357-01p_com
95-030-01p	92-196-01p	Calgene	Tomato	Fruit ripening altered	20 additional FLAVRSAVR lines	95-030-01p	95-030-01p		95-030-01p_com
95-045-01p		Monsanto	Cotton	Glyphosate tolerant	1445, 1698				95-045-01p_com
95-053-01p		Monsanto	Tomato	Fruit ripening altered	8338				95-053-01p_com
95-093-01p		Monsanto	Corn	Lepidopteran resistant	MON 80100				95-093-01p_com
95-145-01p		DeKalb	Corn	Phosphinothricin tolerant	B16				95-145-01p_com
95-179-01p	92-196-01p	Calgene	Tomato	Fruit ripening altered	2 additional FLAVRSAVR lines				95-179-01p_com
95-195-01p		Northrup King	Corn	European Corn Borer resistant	Bt11				95-195-01p_com
95-228-01p		Plant Genetic Systems	Corn	Male sterile	MS3				95-228-01p_com
95-256-01p		Du Pont	Cotton	Sulfonylurea tolerant	19-51a				95-256-01p_com
95-324-01p		Agritope	Tomato	Fruit ripening altered	35 1 N				95-324-01p_com
95-338-01p		Monsanto	Potato	CPB resistant	SBT02-5 & -7, ATBT04-6 & -27, -30, -31, -36				95-338-01p_com
95-352-01p		Asgrow	Squash	CMV, ZYMV, WMV2 resistant	CZW-3				95-352-01p_com

Deregulated GM crops in the US *continued*

Applicant Documents						APHIS Documents			
Petition	Extension of Petition Number ***	Institution	Regulated article	Transgenic Phenotype	Transformation Event or Line	Preliminary EA ****	FR Notice	Risk Asses.	Final EA & Determination
96-017-01p	95-093-01p	Monsanto	Corn	European Corn Borer resistant	MON809 & MON810				96-017-01p_com
96-051-01p		Cornell U	Papaya	PRSV resistant	55-1, 63-1				96-051-01p_com
96-068-01p		AgrEvo	Soybean	Phosphinothricin tolerant	W62, W98, A2704- 12, A2704-21, A5547-35				96-068-01p_com
96-248-01p	92-196-01p	Calgene	Tomato	Fruit ripening altered	1 additional FLAVRSAVR line				96-248-01p_com
96-291-01p		DeKalb	Corn	European Corn Borer resistant	DBT418				96-291-01p_com
96-317-01p		Monsanto	Corn	Glyphosate tolerant & ECB resistant	MON802				96-317-01p_com
97-008-01p		Du Pont	Soybean	Oil profile altered	G94-1, G94-19, G-168				97-008-01p_com
97-013-01p		Calgene	Cotton	Bromoxynil tolerant & Lepidopteran resistant	Events 31807 & 31808				97-013-01p_com
97-099-01p		Monsanto	Corn	Glyphosate tolerant	GA21				97-099-01p_com
97-148-01p		Bejo	Cichorium intybus	Male sterile	RM3-3, RM3-4, RM3-6				97-148-01p_com
97-204-01p		Monsanto	Potato	CPB & PLRV resistant	RBMT21-129 & RBMT21-350				97-204-01p_com
97-205-01p		AgrEvo	Rapeseed	Phosphinothricin tolerant	T45				97-205-01p_com
97-265-01p		AgrEvo	Corn	Phosphinothricin tolerant & Lep. resistant	CBH-351				97-265-01p_com
97-287-01p		Monsanto	Tomato	Lepidopteran resistant	5345				97-287-01p_com
97-336-01p		AgrEvo	Beet	Phosphinothricin tolerant	T-120-7				97-336-01p_com
97-339-01p		Monsanto	Potato	CPB & PVY resistant	RBMT15-101, SEMT15-02, SEMT15-15				97-339-01p_com
97-342-01p		Pioneer	Corn	Male sterile & Phosphinothricin tolerant	676, 678, 680				97-342-01p_com
98-014-01p	96-068-01p	AgrEvo	Soybean	Phosphinothricin tolerant	A5547-127				98-014-01p_com
98-173-01p		Novartis Seeds & Monsanto	Beet	Glyphosate tolerant	GTSB77				98-173-01p_com
98-216-01p		Monsanto	Rapeseed	Glyphosate tolerant	RT73				98-216-01p_com
98-238-01p		AgrEvo	Soybean	Phosphinothricin tolerant	GU262				98-238-01p_com
98-278-01p		AgrEvo	Rapeseed	Phosphinothricin tolerant & Pollination control	MS8 & RF3				98-278-01p_com
98-329-01p		AgrEvo	Rice	Phosphinothricin tolerant	LLRICE06, LLRICE62				98-329-01p_com
98-335-01p		U. of Saskatchewan	Flax	Tolerant to soil residues of sulfonyl urea herbicide	CDC Triffid				98-335-01p_com
98-349-01p	95-228-01p	AgrEvo	Corn	Phosphinothricin tolerant and Male sterile	MS6				98-349-01p_com
99-173-01p	97-204-01p	Monsanto	Potato	PLRV & CPB resistant	RBMT22-82				99-173-01p_com

Deregulated GM crops in the US *continued*

Applicant Documents						APHIS Documents			
Petition	Extension of Petition Number ***	Institution	Regulated article	Transgenic Phenotype	Transformation Event or Line	Preliminary EA ****	FR Notice	Risk Asses.	Final EA & Determination
00-011-01p	97-099-01p	Monsanto	Corn	Glyphosate tolerant	NK603				00-011-01p_com
00-136-01p		Mycogen c/o Dow & Pioneer	Corn	Lepidopteran resistant phosphinothricin tolerant	Line 1507				00-136-01p_com
00-342-01p		Monsanto	Cotton	Lepidopteran resistant	Cotton Event 15985				00-342-01p_com
01-121-01p		Vector	Tobacco	Reduced nicotine	Vector 21-41				01-121-01p_com
01-137-01p		Monsanto	Corn	Corn Rootworm Resistant	MON 863				01-137-01p_com
01-206-01p	98-278-01p	Aventis	Rapeseed	Phosphinothricin tolerant & pollination control	MS1 & RF1/RF2				01-206-01p_com
01-206-02p	97-205-01p	Aventis	Rapeseed	Phosphinothricin tolerant	Topas 19/2				01-206-02p_com
01-324-01p	98-216-01p	Monsanto	Rapeseed	Glyphosate tolerant	RT200				01-324-01p_com
02-042-01p		Aventis	Cotton	Phosphinothricin tolerant	LLCotton25				02-042-01p_com
03-036-01p		Mycogen/Dow	Cotton	Lepidopteran Resistance	281-24-236	03-036-01p_pea	03-036-01p_fr_pc_pet		03-036-01p_com
03-036-02p		Mycogen/Dow	Cotton	Lepidopteran Resistance	3006-210-23	03-036-02p_pea	03-036-02p_fr_pc_pet		03-036-02p_com
03-155-01p		Syngenta	Cotton	Lepidopteran Resistant	COT 102	03-155-01p_pea	03-155-01p_fr_pc_pet		03-155-01p_com
03-181-01p	00-136-01p	Dow	Corn	Lepidopteran Resistant, Glufosinate Tolerant	TO-6275	03-181-01p_pea	03-181-01p_fr_pc_pet		03-181-01p_com
03-323-01p		Monsanto	Sugar Beet	Glyphosate Tolerant	H7-1	03-323-01p_pea	03-323-01p_fr_pc_pet		03-323-01p_com
03-353-01p		Dow	Corn	Corn Rootworm Resistant	59122	03-353-01p_pea	03-353-01p_fr_pc_pet		03-353-01p_com
03-353-01p		Monsanto	Cotton	Glyphosate Tolerant	MON 88913	04-086-01p_pea	04-086-01p_fr_pc_pet		04-086-01p_com
04-110-01p		Monsanto & Forage Genetics	Alfalfa	Glyphosate Tolerant	J101, J163	04-110-01p_pea	04-110-01p_fr_pc_pet		04-110-01p_com

Appendix VI

GM crops with petition pending for deregulation in the US

Applicant Documents						APHIS Documents			
Petition	Extension of Petition Number ***	Institution	Regulated article	Transgenic Phenotype	Transformation Event or Line	Preliminary EA ****	FR Notice	Risk Asses.	Final EA & Determination
03-104-01p		Monsanto & Scotts	Creeping bentgrass	Glyphosate Tolerant	ASR368		03-104-01p_fr_pc_pet	03-104-01p_ra	
04-125-01p		Monsanto	Corn	Corn Rootworm Resistant	88017	04-125-01p_pea	04-125-01p_fr_pc_pet		
04-229-01p		Monsanto	Corn	High Lysine	LY038	04-229-01p_pea	04-229-01p_fr_pc_pet		
04-264-01p		ARS	Plum	Plum Pox Resistant	C5				
04-337-01p		University of Florida	Papaya	Papaya Ringspot Virus Resistant	X17-2				
04-362-01p		Syngenta	Corn	Corn Rootworm Protected	MIR604				
05-280-01p		Syngenta	Corn	Thermostable alpha-amylase	3272				