Chapter 15

Gene Cloning and DNA Analysis in Agriculture

Chapter contents

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Agriculture, or more specifically the cultivation of plants, is the world's oldest biotechnology, with an unbroken history that stretches back at least 10,000 years. Throughout this period humans have constantly searched for improved varieties of their crop plants: varieties with better nutritional qualities, higher yields, or features that aid cultivation and harvesting. During the first few millennia, crop improvements occurred in a sporadic fashion, but in recent centuries new varieties have been obtained by breeding programs of ever increasing sophistication. However, the most sophisticated breeding program still retains an element of chance, dependent as it is on the random merging of parental characteristics in the hybrid offspring that are produced. The development of a new variety of crop plant, displaying a precise combination of desired characteristics, is a lengthy and difficult process.

Gene cloning provides a new dimension to crop breeding by enabling directed changes to be made to the genotype of a plant, circumventing the random processes inherent in conventional breeding. Two general strategies have been used:

- Gene addition, in which cloning is used to alter the characteristics of a plant by providing it with one or more new genes;
- Gene subtraction, in which genetic engineering techniques are used to inactivate one or more of the plant's existing genes.

A number of projects are being carried out around the world, many by biotechnology companies, aimed at exploiting the potential of gene addition and gene subtraction in crop improvement. In this chapter we will investigate a representative selection of

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these projects, and look at some of the problems that must be solved if plant genetic engineering is to gain widespread acceptance in agriculture.

15.1 The gene addition approach to plant genetic engineering

Gene addition involves the use of cloning techniques to introduce into a plant one or more new genes coding for a useful characteristic that the plant lacks. A good example of the technique is provided by the development of plants that resist insect attack by synthesizing insecticides coded by cloned genes.

15.1.1 Plants that make their own insecticides

Plants are subject to predation by virtually all other types of organism—viruses, bacteria, fungi, and animals—but in agricultural settings the greatest problems are caused by insects. To reduce losses, crops are regularly sprayed with insecticides. Most conventional insecticides (e.g., pyrethroids and organophosphates) are relatively non-specific poisons that kill a broad spectrum of insects, not just the ones eating the crop. Because of their high toxicity, several of these insecticides also have potentially harmful side effects for other members of the local biosphere, including in some cases humans. These problems are exacerbated by the need to apply conventional insecticides to the surfaces of plants by spraying, which means that subsequent movement of the chemicals in the ecosystem cannot be controlled. Furthermore, insects that live within the plant, or on the undersurfaces of leaves, can sometimes avoid the toxic effects altogether.

What features would be displayed by the ideal insecticide? Clearly it must be toxic to the insects against which it is targeted, but if possible this toxicity should be highly selective, so that the insecticide is harmless to other insects and is not poisonous to animals and to humans. The insecticide should be biodegradable, so that any residues that remain after the crop is harvested, or which are carried out of the field by rainwater, do not persist long enough to damage the environment. And it should be possible to apply the insecticide in such a way that all parts of the crop, not just the upper surfaces of the plants, are protected against insect attack.

The ideal insecticide has not yet been discovered. The closest we have are the δ-endotoxins produced by the soil bacterium *Bacillus thuringiensis*.

The δ-endotoxins of Bacillus thuringiensis

Insects not only eat plants: bacteria also form an occasional part of their diet. In response, several types of bacteria have evolved defense mechanisms against insect predation, an example being *B. thuringiensis* which, during sporulation, forms intracellular crystalline bodies that contain an insecticidal protein called the δ-endotoxin. The activated protein is highly poisonous to insects, some 80,000 times more toxic than organophosphate insecticides, and is relatively selective, different strains of the bacterium synthesizing proteins effective against the larvae of different groups of insects (Table 15.1).

The δ-endotoxin protein that accumulates in the bacterium is an inactive precursor. After ingestion by the insect, this protoxin is cleaved by proteases, resulting in shorter versions of the protein that display the toxic activity, by binding to the inside of the

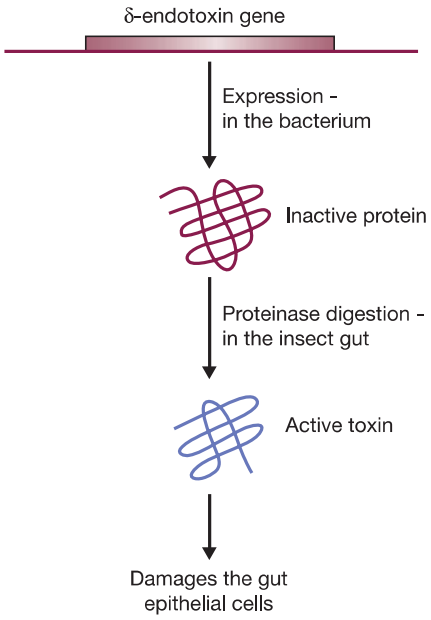
Table 15.1

The range of insects poisoned by the various types of *B. thuringiensis* δ-endotoxins.

δ-ENDOTOXIN TYPE	EFFECTIVE AGAINST
CryI	Lepidoptera (moth and butterfly) larvae
CryII	Lepidoptera and Diptera (two-winged fly) larvae
CryIII	Lepidoptera larvae
CryIV	Diptera larvae
CryV	Nematode worms
CryVI	Nematode worms

Figure 15.1

Mode of action of a δ-endotoxin.



insect's gut and damaging the surface epithelium, so that the insect is unable to feed and consequently starves to death (Figure 15.1). Variation in the structure of these binding sites in different groups of insects is probably the underlying cause of the high specificities displayed by the different types of δ-endotoxin.

B. thuringiensis toxins are not recent discoveries, the first patent for their use in crop protection having been granted in 1904. Over the years there have been several attempts to market them as environmentally friendly insecticides, but their biodegradability acts as a disadvantage because it means that they must be reapplied at regular intervals during the growing season, increasing the farmer's costs. Research has therefore been aimed at developing δ-endotoxins that do not require regular application. One approach is via protein engineering (p. 206), modifying the structure of the toxin so that it is more stable. A second approach is to engineer the crop to synthesize its own toxin.

Cloning a δ-endotoxin gene in maize

Maize is an example of a crop plant that is not served well by conventional insecticides. A major pest is the European corn borer (*Ostrinia nubilialis*), which tunnels into the plant from eggs laid on the undersurfaces of leaves, thereby evading the effects of insecticides applied by spraying. The first attempt at countering this pest by engineering

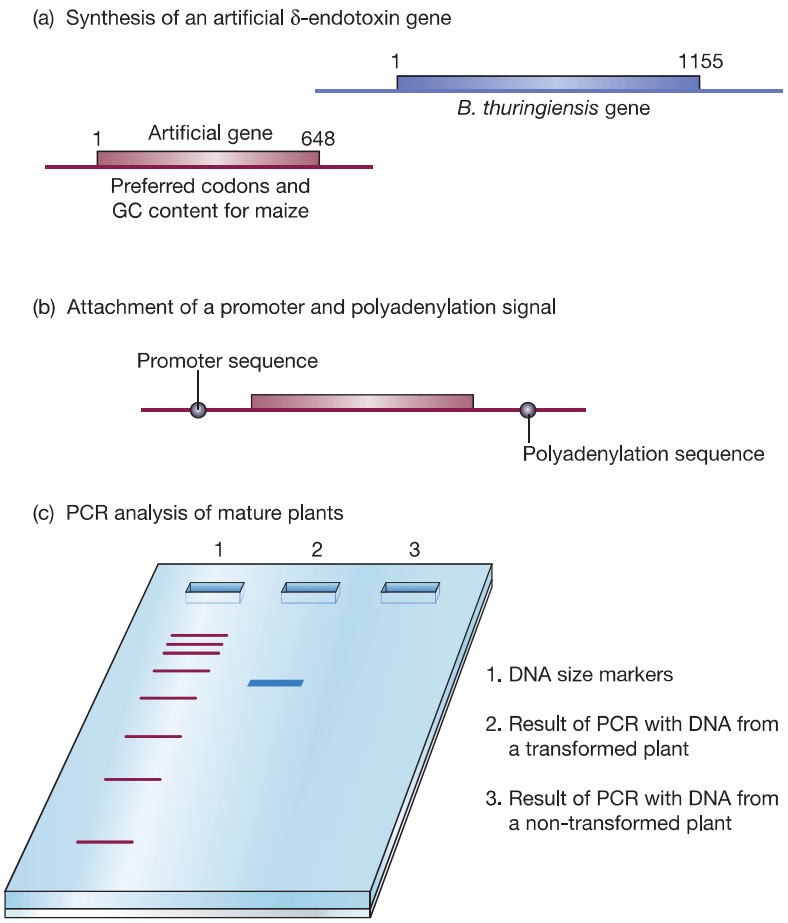


Figure 15.2

Important steps in the procedure used to obtain genetically engineered maize plants expressing an artificial δ-endotoxin gene.

maize plants to synthesize δ-endotoxin was made by plant biotechnologists in 1993, working with the CryIA(b) version of the toxin. The CryIA(b) protein is 1155 amino acids in length, with the toxic activity residing in the segment from amino acids 29–607. Rather than isolating the natural gene, a shortened version containing the first 648 codons was made by artificial gene synthesis. This strategy enabled modifications to be introduced into the gene to improve its expression in maize plants. For example, the codons that were used in the artificial gene were those known to be preferred by maize, and the overall GC content of the gene was set at 65%, compared with the 38% GC content of the native bacterial version of the gene (Figure 15.2a). The artificial gene was ligated into a cassette vector (p. 232) between a promoter and polyadenylation signal from cauliflower mosaic virus (Figure 15.2b), and introduced into maize embryos by bombardment with DNA-coated microprojectiles (p. 85). The embryos were grown into mature plants, and transformants identified by PCR analysis of DNA extracts, using primers specific for a segment of the artificial gene (Figure 15.2c).

The next step was to use an immunological test to determine if δ-endotoxin was being synthesized by the transformed plants. The results showed that the artificial gene was indeed active, but that the amounts of δ-endotoxin being produced varied from plant to plant, from about 250–1750 ng of toxin per mg of total protein. These

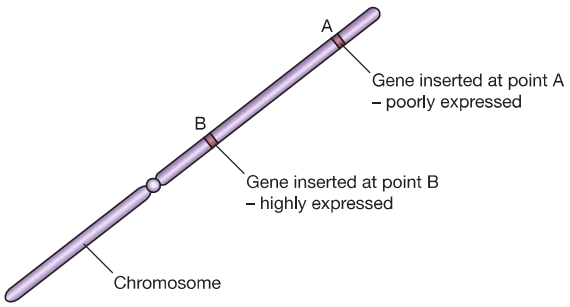


Figure 15.3
Positional effects.

differences were probably due to positional effects, the level of expression of a gene cloned in a plant (or animal) often being influenced by the exact location of the gene in the host chromosomes (Figure 15.3).

Were the transformed plants able to resist the attentions of the corn borers? This was assessed by field trials in which transformed and normal maize plants were artificially infested with larvae and the effects of predation measured over a period of 6 weeks. The criteria that were used were the amount of damage suffered by the foliage of the infested plants, and the lengths of the tunnels produced by the larvae boring into the plants. In both respects the transformed plants gave better results than the normal ones. In particular, the average length of the larval tunnels was reduced from 40.7 cm for the controls to just 6.3 cm for the engineered plants. In real terms, this is a very significant level of resistance.

Cloning δ-endotoxin genes in chloroplasts

One objection that has been raised to the use of GM crops is the possibility that the cloned gene might escape from the engineered plant and become established in a weed species. From a biological viewpoint, this is an unlikely scenario as the pollen produced by a plant is usually only able to fertilize the ovary of a plant of the same species, so transfer of a gene from a crop to a weed is highly unlikely. However, one way of making such transfer totally impossible would be to place the cloned gene not in the nucleus but in the plant's chloroplasts. A transgene located in the chloroplast genome cannot escape via pollen for the simple reason that pollen does not contain chloroplasts.

Synthesis of δ-endotoxin protein in transgenic chloroplasts has been achieved in an experimental system with tobacco. The CryIIA(a2) gene was used, which codes for a protein that has a broader toxicity spectrum than the CryIA toxins, killing the larvae of two-winged flies as well as lepidopterans (see Table 15.1). In the *B. thuringiensis* genome, the CryIIA(a2) gene is the third gene in a short operon, the first two genes of which code for proteins that help to fold and process the δ-endotoxin (Figure 15.4). One advantage of using chloroplasts as the sites of recombinant protein synthesis is that the gene expression machinery of chloroplasts, being related to that of bacteria (because chloroplasts were once free-living prokaryotes), is able to express all the genes in an operon. In contrast, each gene that is placed in a plant (or animal) nuclear genome must be cloned individually, with its own promoter and other expression signals, which makes it very difficult to introduce two or more genes at the same time.

Biolistics (p. 85) was used to introduce the CryIIA(a2) operon into tobacco leaf cells. Insertion into the chloroplast genome was ensured by attaching chloroplast DNA

Figure 15.4
The CryIIA(a2) operon.

sequences to the operon (p. 119), and by rigorously selecting for the kanamycin resistance marker by placing leaf segments on agar containing kanamycin for up to 13 weeks. Transgenic shoots growing out of the leaf segments were then placed on a medium that induced root formation, and plants grown.

The amounts of CryIIA(a2) protein produced in the tissues of these GM plants was quite remarkable, the toxin making up over 45% of the total soluble protein, more than previously achieved in any plant cloning experiment. This high level of expression almost certainly results from the combined effects of the high copy number for the transgene (there being many chloroplast genomes per cell, compared with just two copies of the nuclear genome) and the presence in the chloroplasts of the two helper proteins coded by the other genes in the CryIIA(a2) operon. As might be anticipated, the plants proved to be extremely toxic to susceptible insect larvae. Five days after being placed on the GM plants, all cotton bollworm and beet armyworm larvae were dead, with appreciable damage being visible only on the leaves of the plants exposed to armyworms, which have a relatively high natural resistance to δ-endotoxins. The presence of large amounts of toxin in the leaf tissues appeared not to affect the plants themselves, the GM tobacco being undistinguishable from non-GM plants when factors such as growth rates, chlorophyll content, and level of photosynthesis were considered. Attempts to repeat this experiment with maize, cotton, and other more useful crops have been hampered by the difficulties in achieving chloroplast transformation with plants other than tobacco (see p. 119).

Countering insect resistance to δ-endotoxin crops

It has long been recognized that crops synthesizing δ-endotoxins might become ineffective after a few seasons due to the build-up of resistance among the insect populations feeding on the crops. This would be a natural consequence of exposing these populations to high amounts of toxins and, of course, could render the GM plants no better than the non-GM versions after a just a few years. Various strategies have been proposed to prevent the development of δ-endotoxin resistant insects. One of the first to be suggested was to develop crops expressing both the CryI and CryII genes, the rationale being that as these toxins are quite different it would be difficult for an insect population to develop resistance to both types (Figure 15.5a). Whether or not this is a sound argument is not yet clear. Most examples of δ-endotoxin resistance that have been documented have not been broad spectrum: for example, the CryIIA(a2) tobacco plants described above were equally poisonous to cotton budworms that were or were not resistant to CryIA(b). However, some strains of meal moth larvae exposed to plants containing the CryIA(c) toxin have acquired a resistance that also provides protection against the CryII toxins. In any case, it would be risky to base a counter-resistance strategy on assumed limitations to the genetic potential of the insect pests.

An alternative might be to engineer toxin production in such a way that synthesis occurs only in those parts of the plant that need protection. For example, in a crop such as maize, some damage to the non-fruiting parts of the plant could be tolerated if this did not affect the production of cobs (Figure 15.5b). If expression of the toxin only occurred late in the plant life cycle, when the cobs are developing, then overall exposure of the insects to the toxin might be reduced without any decrease in the value of the crop. However, this strategy might delay the onset of resistance, but it is unlikely to avoid it altogether.

A third strategy is to mix GM plants with non-GM ones, so that each field contains plants that the insects can feed on without being exposed to the toxin produced by the

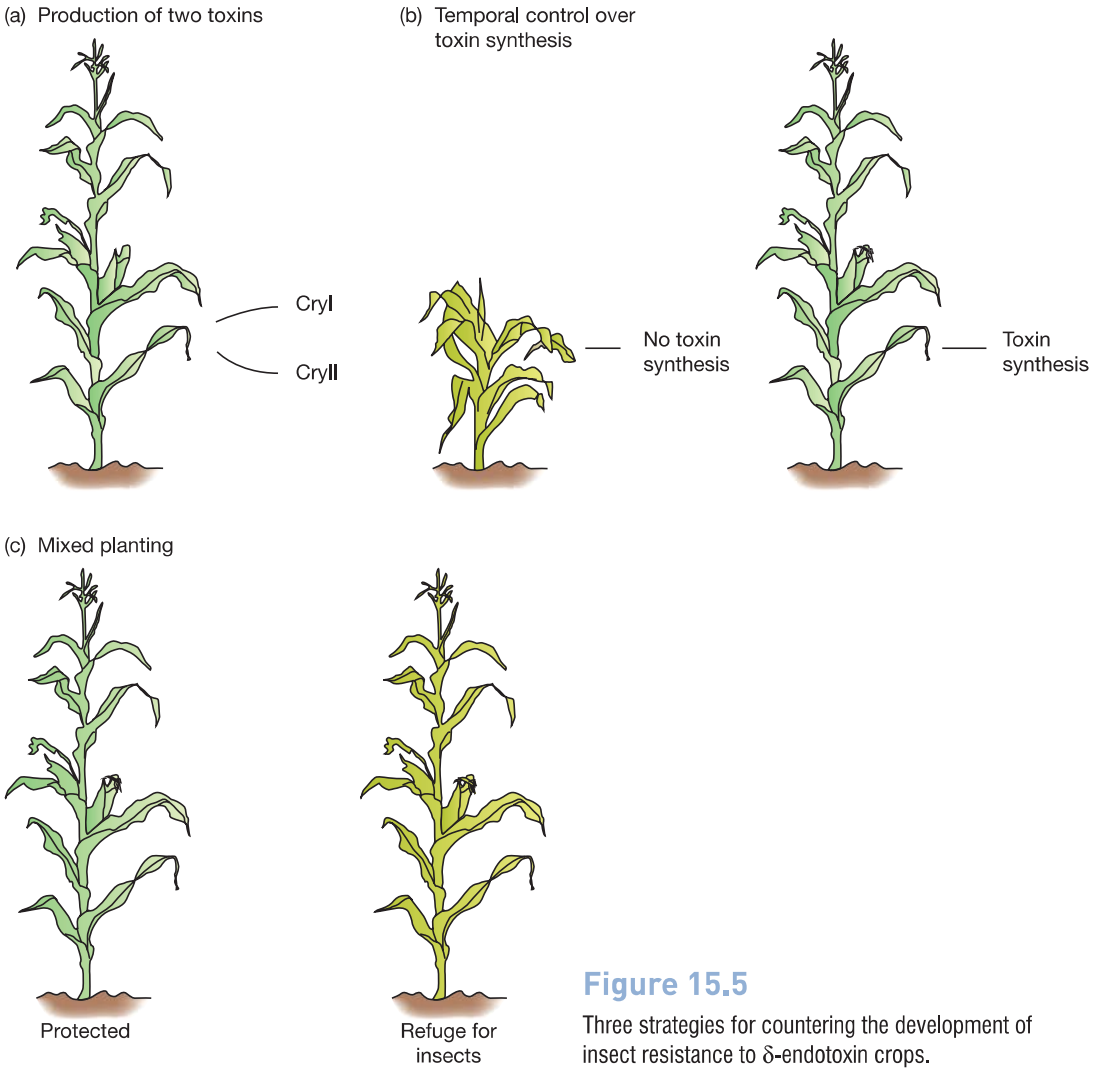


Figure 15.5
Three strategies for countering the development of insect resistance to δ-endotoxin crops.

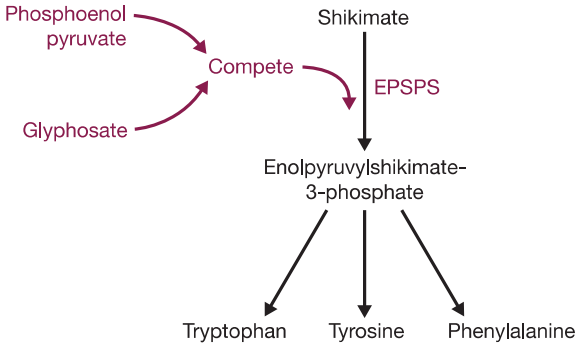
engineered versions (Figure 15.5c). These non-GM plants would act as a refuge for the insects, ensuring that the insect population continually includes a high proportion of non-resistant individuals. As all the δ-endotoxin resistance phenotypes so far encountered are recessive, heterozygotes arising from a mating between a susceptible insect and a resistant partner would themselves be susceptible, continually diluting the proportion of resistant insects in the population. Trials have been carried out, and theoretical models have been examined, to identify the most effective mixed planting strategies. In practice, success or failure would depend to a very large extent on the farmers who grow the crops, these farmers having to adhere to the precise planting strategy dictated by the scientists, despite the resulting loss in productivity due to the damage suffered by the non-GM plants. Again, this introduces an element of risk. The success of GM projects with plants clearly depends on much more that the cleverness of the genetic engineers.

15.1.2 Herbicide resistant crops

Although δ-endotoxin production has been engineered in crops as diverse as maize, cotton, rice, potato, and tomato, these plants are not the most widespread GM crops

Figure 15.6

Glyphosate competes with phosphoenol pyruvate in the EPSPS catalyzed synthesis of enolpyruvylshikimate-3-phosphate, and hence inhibits synthesis of tryptophan, tyrosine, and phenylalanine.



grown today. In commercial terms the most important transgenic plants are those that have been engineered to withstand the herbicide glyphosate. This herbicide, which is widely used by farmers and horticulturists, is environmentally friendly, as it is non-toxic to insects and to animals and has a short residence time in soils, breaking down over a period of a few days into harmless products. However, glyphosate kills all plants, both weeds and crop species, and so has to be applied to fields very carefully in order to prevent the growth of weeds without harming the crop itself. GM crops that are able to withstand the effects of glyphosate are therefore desirable as they would enable a less rigorous and hence less expensive herbicide application regime to be followed.

"Roundup Ready" crops

The first crops to be engineered for glyphosate resistance were produced by Monsanto Co. and called "Roundup Ready", reflecting the trade name of the herbicide. These plants contain modified genes for the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS), which converts shikimate and phosphoenol pyruvate (PEP) into enolpyruvylshikimate-3-phosphate, an essential precursor for synthesis of the aromatic amino acids tryptophan, tyrosine, and phenylalanine (Figure 15.6). Glyphosate competes with PEP for binding to the enzyme surface, thereby inhibiting synthesis of enolpyruvylshikimate-3-phosphate and preventing the plant from making the three amino acids. Without these amino acids, the plant quickly dies.

Initially, genetic engineering was used to generate plants that made greater than normal amounts of EPSPS, in the expectation that these would be able to withstand higher doses of glyphosate than non-engineered plants. However, this approach was unsuccessful because, although engineered plants that made up to 80 times the normal amount of EPSPS were obtained, the resulting increase in glyphosate tolerance was not sufficient to protect these plants from herbicide application in the field.

A search was therefore carried out for an organism whose EPSPS enzyme is resistant to glyphosate inhibition and whose EPSPS gene might therefore be used to confer resistance on a crop plant. After testing the genes from various bacteria, as well as mutant forms of *Petunia* that displayed glyphosate resistance, the EPSPS gene from *Agrobacterium* strain CP4 was chosen, because of its combination of high catalytic activity and high resistance to the herbicide. EPSPS is located in the plant chloroplasts, so the *Agrobacterium* EPSPS gene was cloned in a Ti vector as a fusion protein with a leader sequence that would direct the enzyme across the chloroplast membrane and into the organelle. Biolistics was used to introduce the recombinant vector into soybean callus culture. After regeneration, the GM plants were found to have a threefold increase in herbicide resistance.

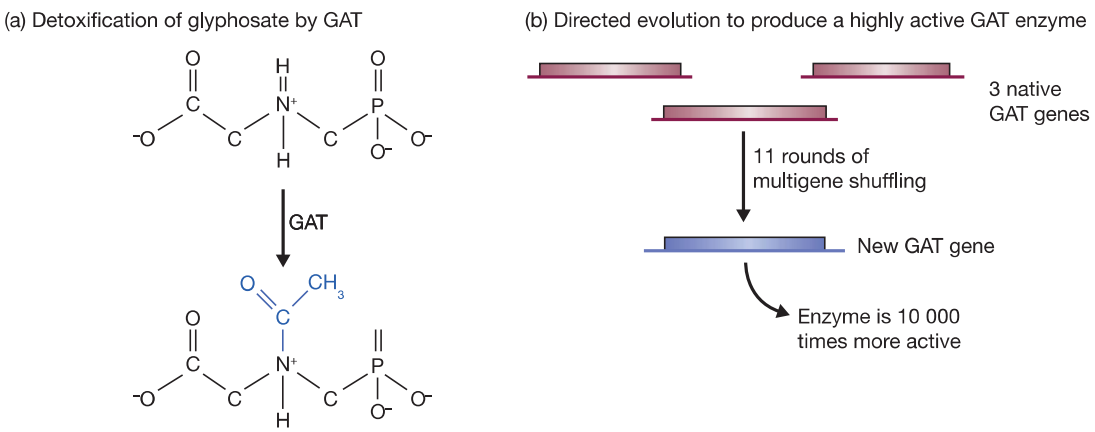


Figure 15.7

Use of glyphosate *N*-acetyltransferase to generate plants that detoxify glyphosate. (a) GAT detoxifies glyphosate by adding an acetyl group (shown in blue). (b) Creation of a highly active GAT enzyme by multigene shuffling.

A new generation of glyphosate resistant crops

Roundup Ready versions of a variety of crops have been produced in recent years, and several of these, in particular soybean and maize, are grown routinely in the USA and other parts of the world. However, these plants do not actually destroy glyphosate, which means that the herbicide can accumulate in the plant tissues. Glyphosate is not poisonous to humans or other animals, so the use of such plants as food or forage should not be a concern, but accumulation of the herbicide can interfere with reproduction of the plant. The degree of resistance displayed by Roundup Ready crops has also been found to be too low to provide a major economic benefit with some crops, notably wheat.

Until recently, there had been only a few scattered reports of organisms capable of actively degrading glyphosate. However, searches of microbial collections have revealed that this property is relatively common among bacteria of the genus *Bacillus*, which possess an enzyme, now called glyphosate *N*-acetyltransferase (GAT), which detoxifies glyphosate by attaching an acetyl group to the herbicide molecule (Figure 15.7a). The most active detoxifier known is a strain of *B. licheniformis*, but even this bacterium detoxifies glyphosate at rates that are too low to be of value if transferred to a GM crop.

Is it possible to increase the activity of the GAT synthesized by *B. licheniformis*? The discovery that the bacterium possesses three related genes for this enzyme pointed a way forward. A type of **directed evolution** called **multigene shuffling** was used. Multigene shuffling involves taking parts of each member of a multigene family and reassembling these parts to create new gene variants. At each stage of the process, the most active genes are identified by cloning all variants in *E. coli* and assaying the recombinant colonies for GAT activity. The most active genes are then used as the substrates for the next round of shuffling. After 11 rounds, a gene specifying a GAT with 10,000 times the activity of the enzymes present in the original *B. licheniformis* strain was obtained (Figure 15.7b). This gene was introduced into maize, and the resulting GM plants were found to tolerate levels of glyphosate six times higher than the amount normally used by farmers to control weeds, without any reduction in the productivity of the plant. This new way of engineering glyphosate resistance is currently being examined in greater detail to determine if it presents a real alternative to Roundup Ready crops.

Table 15.2

Examples of gene addition projects with plants.

GENE FOR	SOURCE ORGANISM	CHARACTERISTIC CONFERRED ON MODIFIED PLANTS
δ-Endotoxin	*B. thuringiensis*	Insect resistance
Proteinase inhibitors	Various legumes	Insect resistance
Chitinase	Rice	Fungal resistance
Glucanase	Alfalfa	Fungal resistance
Ribosome-inactivating protein	Barley	Fungal resistance
Ornithine carbamyltransferase	*Pseudomonas syringae*	Bacterial resistance
RNA polymerase, helicase	Potato leafroll luteovirus	Virus resistance
Satellite RNAs	Various viruses	Virus resistance
Virus coat proteins	Various viruses	Virus resistance
2′–5′-Oligoadenylate synthetase	Rat	Virus resistance
Acetolactate synthase	*Nicotiana tabacum*	Herbicide resistance
Enolpyruvylshikimate-3-phosphate synthase	*Agrobacterium* spp.	Herbicide resistance
Glyphosate oxidoreductase	*Ochrobactrum anthropi*	Herbicide resistance
Glyphosate *N*-acetyltransferase	*B. licheniformis*	Herbicide resistance
Nitrilase	*Klebsiella ozaenae*	Herbicide resistance
Phosphinothricin acetyltransferase	*Streptomyces* spp.	Herbicide resistance
Phosphatidylinositol-specific phospholipase C	Maize	Drought tolerance
Barnase ribonuclease inhibitor	*Bacillus amyloliquefaciens*	Male sterility
DNA adenine methylase	*E. coli*	Male sterility
Methionine-rich protein	Brazil nuts	Improved sulphur content
1-Aminocyclopropane-1-carboxylic acid deaminase	Various	Modified fruit ripening
S-Adenosylmethionine hydrolase	Bacteriophage T3	Modified fruit ripening
Monellin	*Thaumatococcus danielli*	Sweetness
Thaumatin	*T. danielli*	Sweetness
Acyl carrier protein thioesterase	*Umbellularia californica*	Modified fat/oil content
Delta-12 desaturase	*Glycine max*	Modified fat/oil content
Dihydroflavanol reductase	Various flowering plants	Modified flower color
Flavonoid hydroxylase	Various flowering plants	Modified flower color

15.1.3 Other gene addition projects

GM crops that synthesize δ-endotoxins or glyphosate resistance enzymes are by no means the only examples of plants engineered by gene addition. Examples of other gene addition projects are listed in Table 15.2. These projects include an alternative means of conferring insect resistance, using genes coding for proteinase inhibitors, small polypeptides that disrupt the activities of enzymes in the insect gut, preventing or slowing growth. Proteinase inhibitors are produced naturally by several types of plant, notably legumes such as cowpeas and common beans, and their genes have been successfully transferred to other crops which do not normally make significant amounts of these proteins. The inhibitors are particularly effective against beetle larvae that feed on seeds, and so may be a better alternative than δ-endotoxin for plants whose seeds are stored for long periods. Other projects are exploring the use of genetic modification to improve the nutritional quality of crop plants, for example by increasing the content of essential amino acids or by changing the plant biochemistry so that more of the available nutrients can be utilized during digestion by humans or animals. Finally, in a different sphere of commercial activity, ornamental plants with unusual flower colors

are being produced by transferring genes for enzymes involved in pigment production from one species to another.

15.2 Gene subtraction

The second way of changing the genotype of a plant is by gene subtraction. This term is a misnomer, as the modification does not involve the actual removal of a gene, merely its inactivation. There are several possible strategies for inactivating a single, chosen gene in a living plant, the most successful so far in practical terms being the use of antisense RNA (p. 260).

15.2.1 Antisense RNA and the engineering of fruit ripening in tomato

To illustrate how antisense RNA has been used in plant genetic engineering, we will examine how tomatoes with delayed ripening have been produced. This is an important example of plant genetic modification as it resulted in one of the first GM foodstuffs to be approved for sale to the general public.

Commercially grown tomatoes and other soft fruits are usually picked before they are completely ripe, to allow time for the fruits to be transported to the marketplace before they begin to spoil. This is essential if the process is to be economically viable, but there is a problem in that most immature fruits do not develop their full flavor if they are removed from the plant before they are fully ripe. The result is that mass-produced tomatoes often have a bland taste, which makes them less attractive to the consumer. Antisense technology has been used in two ways to genetically engineer tomato plants so that the fruit ripening process is slowed down. This enables the grower to leave the fruits on the plant until they ripen to the stage where the flavor has fully developed, there still being time to transport and market the crop before spoilage sets in.

Using antisense RNA to inactivate the polygalacturonase gene

The timescale for development of a fruit is measured as the number of days or weeks after flowering. In tomato, this process takes approximately eight weeks from start to finish, with the color and flavor changes associated with ripening beginning after about six weeks. At this time a number of genes involved in the later stages of ripening are switched on, including one coding for the polygalacturonase enzyme (Figure 15.8). This enzyme slowly breaks down the polygalacturonic acid component of the cell walls in the fruit pericarp, resulting in a gradual softening. The softening makes the fruit palatable, but if taken too far results in a squashy, spoilt tomato, attractive only to students with limited financial resources.

Partial inactivation of the polygalacturonase gene should increase the time between flavor development and spoilage of the fruit. To test this hypothesis, a 730 bp restriction fragment was obtained from the 5′ region of the normal polygalacturonase gene, representing just under half of the coding sequence (Figure 15.9). The orientation of the fragment was reversed, a cauliflower mosaic virus promoter was ligated to the start of the sequence, and a plant polyadenylation signal attached to the end. The construction was then inserted into the Ti plasmid vector pBIN19 (p. 116). Once inside the plant, transcription from the cauliflower mosaic virus promoter should result in synthesis of an antisense RNA complementary to the first half of the polygalacturonase mRNA.

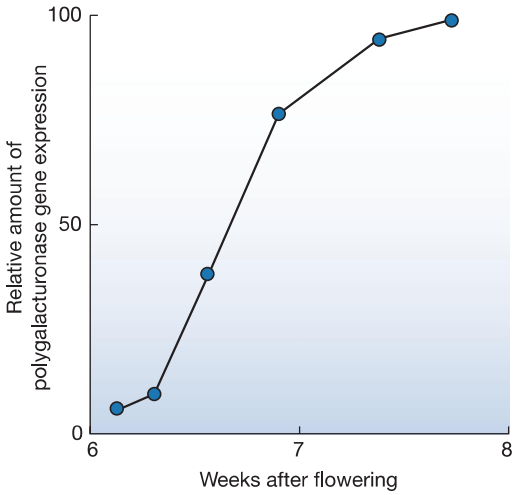


Figure 15.8
The increase in polygalacturonase gene expression seen during the later stages of tomato fruit ripening.

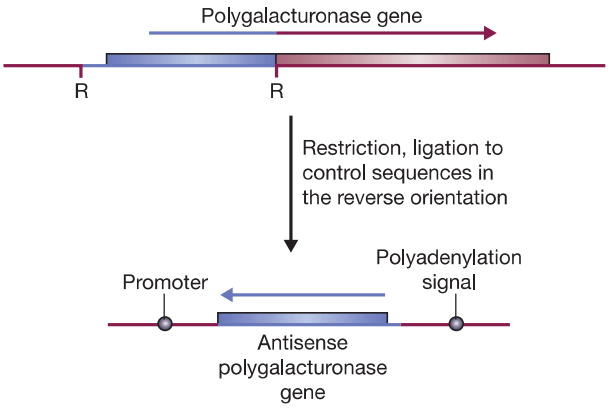


Figure 15.9
Construction of an antisense polygalacturonase "gene". R = restriction site.

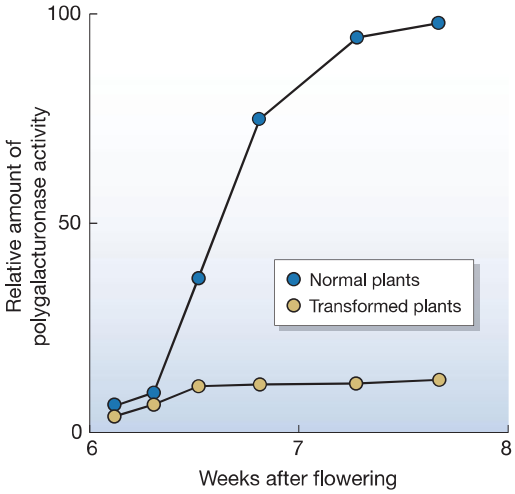
Previous experiments with antisense RNA had suggested that this would be sufficient to reduce or even prevent translation of the target mRNA.

Transformation was carried out by introducing the recombinant pBIN19 molecules into *Agrobacterium tumefaciens* bacteria and then allowing the bacteria to infect tomato stem segments. Small amounts of callus material collected from the surfaces of these segments were tested for their ability to grow on an agar medium containing kanamycin (remember that pBIN19 carries a gene for kanamycin resistance; see Figure 7.14). Resistant transformants were identified and allowed to develop into mature plants.

The effect of antisense RNA synthesis on the amount of polygalacturonase mRNA in the cells of ripening fruit was determined by northern hybridization with a single-stranded DNA probe specific for the sense mRNA. These experiments showed that ripening fruit from transformed plants contained less polygalacturonase mRNA than the fruits from normal plants. The amounts of polygalacturonase enzyme produced in the ripening fruits of transformed plants were then estimated from the intensities of the relevant bands after separation of fruit proteins by polyacrylamide gel electrophoresis, and by directly measuring the enzyme activities in the fruits. The results showed that less enzyme was synthesized in transformed fruits (Figure 15.10). Most importantly, the transformed fruits, although undergoing a gradual softening, could be stored for a prolonged period before beginning to spoil. This indicated that the antisense RNA had not completely inactivated the polygalacturonase gene, but had nonetheless produced a sufficient reduction in gene expression to delay the ripening process as desired. The

Figure 15.10

The differences in polygalacturonase activity in normal tomato fruits and in fruits expressing the antisense polygalacturonase gene.



GM tomatoes—marketed under the trade name "FlavrSavr"—were one of the first genetically engineered plants to be approved for sale to the public, first appearing in supermarkets in 1994.

Using antisense RNA to inactivate ethylene synthesis

The main trigger that switches on the genes involved in the later stages of tomato ripening is ethylene which, despite being a gas, acts as a hormone in many plants. A second way of delaying fruit ripening would therefore be to engineer plants so that they do not synthesize ethylene. Fruits on these plants would develop as normal for the first six weeks, but would be unable to complete the ripening process. The unripe fruit could therefore be transported to the marketplace without any danger of the crop spoiling. Before selling to the consumer, or conversion into paste or some other product, artificial ripening would be induced by spraying the tomatoes with ethylene.

The penultimate step in the ethylene synthesis pathway is conversion of *S*-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (ACC), which is the immediate precursor for ethylene. This step is catalyzed by an enzyme called ACC synthase. As with polygalacturonase, ACC synthase inactivation was achieved by cloning into tomato a truncated version of the normal ACC synthase gene, inserted into the cloning vector in the reverse orientation, so that the construct would direct synthesis of an antisense version of the ACC synthase mRNA. After regeneration, the engineered plants were grown to the fruiting stage and found to make only 2% of the amount of ethylene produced by non-engineered plants. This reduction was more than sufficient to prevent the fruit from completing the ripening process. These tomatoes have been marketed as the "Endless Summer" variety.

15.2.2 Other examples of the use of antisense RNA in plant genetic engineering

In general terms, the applications of gene subtraction in plant genetic engineering are probably less broad than those of gene addition. It is easier to think of useful characteristics that a plant lacks and which might be introduced by gene addition, than it is to identify disadvantageous traits that the plant already possesses and which could be removed by gene subtraction. There are, however, a growing number of plant

Table 15.3

Examples of gene subtraction projects with plants.

TARGET GENE	MODIFIED CHARACTERISTIC
Polygalacturonase	Delay of fruit spoilage in tomato
1-Aminocyclopropane-1-carboxylic acid synthase	Modified fruit ripening in tomato
Polyphenol oxidase	Prevention of discoloration in fruits and vegetables
Starch synthase	Reduction of starch content in vegetables
Delta-12 desaturase	High oleic acid content in soybean
Chalcone synthase	Modification of flower color in various decorative plants
1D-*myo*-inositol 3-phosphate synthase	Reduction of indigestible phosphorus content of rice grains

biotechnology projects based on gene subtraction (Table 15.3), and the approach is likely to increase in importance as the uncertainties that surround the underlying principles of antisense technology are gradually resolved.

15.3 Problems with genetically modified plants

Ripening-delayed tomatoes produced by gene subtraction were among the first genetically modified whole foods to be approved for marketing. Partly because of this, plant genetic engineering has provided the battleground on which biotechnologists and other interested parties have fought over the safety and ethical issues that arise from our ability to alter the genetic make-up of living organisms. A number of the most important questions do not directly concern genes and the expertise needed to answer them will not be found in this book. For example, we cannot discuss in an authoritative fashion the possible impact, good or otherwise, that GM crops might have on local farming practices in the developing world. However, we can, and should, look at the biological issues.

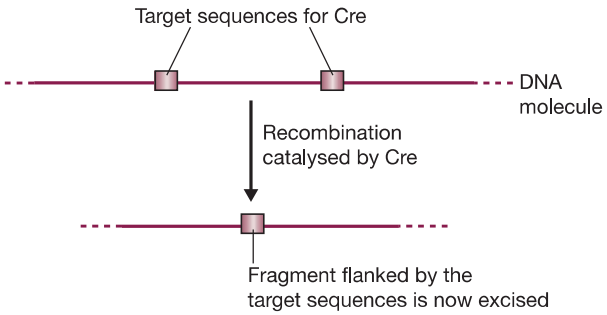
15.3.1 Safety concerns with selectable markers

One of the main areas of concern to emerge from the debate over genetically modified tomatoes is the possible harmful effects of the marker genes used with plant cloning vectors. Most plant vectors carry a copy of a gene for kanamycin resistance, enabling transformed plants to be identified during the cloning process. The kan^R gene, also called *nptII*, is bacterial in origin and codes for the enzyme neomycin phosphotransferase II. This gene and its enzyme product are present in all cells of an engineered plant. The fear that neomycin phosphotransferase might be toxic to humans has been allayed by tests with animal models, but two other safety issues remain:

- Could the kan^R gene contained in a genetically modified foodstuff be passed to bacteria in the human gut, making these resistant to kanamycin and related antibiotics?
- Could the kan^R gene be passed to other organisms in the environment, and would this result in damage to the ecosystem?

Neither question can be fully answered with our current knowledge. It can be argued that digestive processes would destroy all the kan^R genes in a genetically modified food before they could reach the bacterial flora of the gut, and that, even if a gene did avoid

Figure 15.11
DNA excision by the Cre recombinase enzyme.



destruction, the chances of it being transferred to a bacterium would be very small. Nevertheless, the risk factor is not zero. Similarly, although experiments suggest that growth of genetically modified plants would have a negligible effect on the environment, as *kan*^{R} genes are already common in natural ecosystems, the future occurrence of some unforeseen and damaging event cannot be considered an absolute impossibility.

The fears surrounding the use of *kan*^{R} and other marker genes have prompted biotechnologists to devise ways of removing these genes from plant DNA after the transformation event has been verified. One of the strategies makes use of an enzyme from bacteriophage P1, called Cre, which catalyzes a recombination event that excises DNA fragments flanked by specific 34 bp recognition sequences (Figure 15.11). To use this system the plant is transformed with two cloning vectors, the first carrying the gene being added to the plant along with its *kan*^{R} selectable marker gene surrounded by the Cre target sequences, and the second carrying the Cre gene. After transformation, expression of the Cre gene results in excision of the *kan*^{R} gene from the plant DNA.

What if the Cre gene is itself hazardous in some way? This is immaterial as the two vectors used in the transformation would probably integrate their DNA fragments into different chromosomes, so random segregation during sexual reproduction would result in first generation plants that contained one integrated fragment but not the other. A plant that contains neither the Cre gene nor the *kan*^{R} selectable marker, but does contain the important gene that we wished to add to the plant's genome, can therefore be obtained.

15.3.2 The terminator technology

The Cre recombination system also underlies one of the most controversial aspects of plant genetic engineering, the so-called **terminator technology**. This is one of the processes by which the companies who market GM crops attempt to protect their financial investment by ensuring that farmers must buy new seed every year, rather than simply collecting seed from the crop and sowing this second generation seed the following year. In reality, even with conventional crops, mechanisms have been devised to ensure that second generation seed cannot be grown by farmers, but the general controversies surrounding GM crops have placed the terminator technology in the public eye.

The terminator technology centers on the gene for ribosome inactivating protein (RIP). The ribosome inactivating protein blocks protein synthesis by cutting one of the ribosomal RNA molecules into two segments (Figure 15.12a). Any cell in which the ribosome inactivating protein is active will quickly die. In GM plants that utilize the terminator system, the RIP gene is placed under control of a promoter that is active only during embryo development. The plants therefore grow normally but the seeds that they produce are sterile.

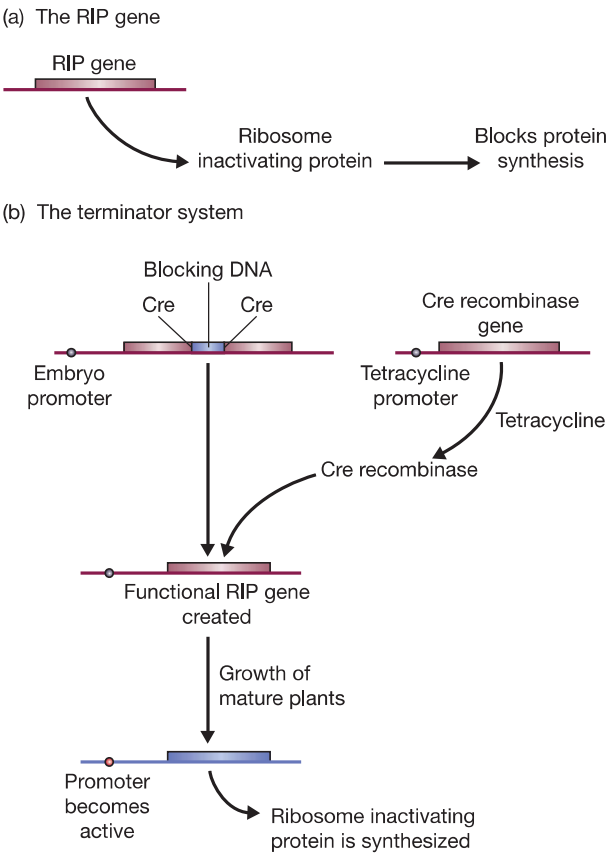


Figure 15.12
The terminator technology. (a) The RIP gene codes for a protein that blocks protein synthesis. (b) The system that is used to allow first generation seeds to be produced.

How are the first generation seeds, those sold to farmers, obtained? To begin with, the RIP gene is non-functional because it is disrupted by a segment of non-RIP DNA (Figure 15.12b). However, this DNA is flanked by the 34 bp recognition sequences for the Cre recombinase. In these plants the gene for the Cre recombinase is placed under control of a promoter that is switched on by tetracycline. Once seeds have been obtained, the supplier activates the Cre recombinase by placing the seeds in a tetracycline solution. This removes the blocking DNA from the RIP gene, which becomes functional but remains silent until its own promoter becomes active during embryogenesis.

15.3.3 The possibility of harmful effects on the environment

A second area of concern regarding genetically modified plants is that their new gene combinations might harm the environment in some way. These concerns have to be addressed individually for each type of GM crop, as different engineered genes might have different impacts. We will examine the work that has been carried out to assess whether it is possible that herbicide resistant plants, one of the two examples of gene addition that we studied earlier in this chapter, can have a harmful effect. As these are the most widely grown GM crops, they have been subject to some of the most comprehensive environmental studies. In particular, in 1999, the UK Government commissioned an independent investigation into how herbicide resistant crops, whose growth in the UK was not at that time permitted, might affect the abundance and diversity of farmland wildlife.

After delays due to activists attempting to prevent the work from being carried out, the UK research team reported their findings in 2003. The study involved 273 field trials throughout England, Wales, and Scotland, and included glyphosate resistant sugar beet as well as maize and spring rape engineered for resistance to a second herbicide, glufosinate-ammonium. The results, as summarized in the official report (see Burke (2003) in *Further Reading*), were as follows:

> The team found that there were differences in the abundance of wildlife between GM crop fields and conventional crop fields. Growing conventional beet and spring rape was better for many groups of wildlife than growing GM beet and spring rape. There were more insects, such as butterflies and bees, in and around the conventional crops because there were more weeds to provide food and cover. There were also more weed seeds in conventional beet and spring rape crops than in their GM counterparts. Such seeds are important in the diets of some animals, particularly some birds. In contrast, growing GM maize was better for many groups of wildlife than conventional maize. There were more weeds in and around the GM crops, more butterflies and bees around at certain times of the year, and more weed seeds. The researchers stress that the differences they found do not arise just because the crops have been genetically modified. They arise because these GM crops give farmers new options for weed control. That is, they use different herbicides and apply them differently. The results of this study suggest that growing such GM crops could have implications for wider farmland biodiversity. However, other issues will affect the medium- and long-term impacts, such as the areas and distribution of land involved, how the land is cultivated and how crop rotations are managed. These make it hard for researchers to predict the medium- and large-scale effects of GM cropping with any certainty. In addition, other management decisions taken by farmers growing conventional crops will continue to impact on wildlife.

Further reading

FURTHER READING

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